The Application of Subtractive Hybridisation to Detect Intra-Species Variation in *Campylobacter jejuni*

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List of Abbreviations

A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
bp	base pairs
cDNA	complementary deoxyribose nucleic acid
CFU	colony forming units
DCA	deoxycholic acid
(d)dH_2O	(double) distilled water
DNA	deoxyribose nucleic acid
dNTP	2´-deoxy nucleotide triphosphate
h	hour(s)
kb	kilobase pairs
kPa	kilopascals
min	minute(s)
mRNA	messenger RNA
ORF	open reading frame
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
RNA	ribose nucleic acid
RT-PCR	reverse-transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
sec	second(s)
spp.	species
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
$\times g$	times gravity

Abstract

Significant intra-species variation is evident in many pathogenic bacteria. Possession of a specific gene or cluster of genes can lead to the greater pathogenicity of that strain. *Campylobacter jejuni* is seemingly ubiquitous in the environment and is frequently isolated from many species of birds, cattle, sheep and other animals, as well as water. Molecular typing studies suggest *C. jejuni* populations are heterogenous, with the emergence of some stable clones. Strains of *C. jejuni* are naturally competent with a propensity for intra-species DNA uptake. Virulence determinants and disease mechanisms are poorly understood and research with regards to the pathogenic potential of this organism is contradictory.

The primary objective of this study was to determine whether all strains of *C. jejuni* are equally pathogenic to humans. A PCR-based subtractive hybridisation method was applied to detect differences between two strains of *C. jejuni*, NCTC11168 (tester) and F38011 (driver). Using this method, a total of eleven DNA fragments were isolated and sequenced. Blast searches revealed all fragments corresponded to the tester strain NCTC11168. The fragments showed significant amino acid identities with the flagella hook protein (FlgE), an A/G-specific adenine glycosylase and a L-serine dehydratase. Nucleotide sequences corresponding to *flgE* were further analysed and one base pair substitution was observed between the tester and the driver (F38011). This study demonstrates that the PCR-based subtractive hybridisation method was reproducible and has the potential to identify fragments corresponding to genes with relevance to virulence. However, this method requires optimisation for the efficient isolation of strain differences in *C. jejuni*. The feasibility of the application of mRNA approaches and RAPD-PCR to detect intra-species variation in *C. jejuni* were also investigated. Difficulties encountered with mRNA detection excluded further investigation of mRNA-based techniques. RAPD-PCR was not sufficiently reproducible to continue analysis of the significance of differences in RAPD profiles in relation to the pathogenic potential of *C. jejuni*.

Chapter 1

Introduction

1.1 Description of the genus

1.1.1 Basic morphology and physiology

The genus *Campylobacter* is part of the family *Campylobacteraceae* and includes 18 species and subspecies (Nachamkin, 1995). *Campylobacter* are Gram-negative, non-spore-forming, motile, curved or spiral rods, 0.2-0.9 μm wide and 0.5-5 μm long. The thermotolerant species, *C. jejuni, C. coli, C. lari* and *C. upsaliensis,* are the most clinically relevant and have an optimal growth temperature of 42°C. Most *Campylobacter* spp. require a microaerophillic atmosphere containing 5% O_2, 10% CO_2 and 85% N_2, for optimal recovery (Nachamkin, 1995). *C. jejuni* and *C. coli* are the most important human pathogens, with *C. jejuni* responsible for approximately 80-90% of enteric *Campylobacter* infections (Ketley, 1997).

1.1.2 Genetics of *Campylobacter* spp.

The small genome size of *Campylobacter* spp. (approximately one third of the *Escherichia coli* genome) is consistent with their requirement for complex media for growth and their inability to degrade complex substances or ferment carbohydrates (Taylor, 1992; Ketley, 1997). The genome is characterised by a very low GC ratio of approximately 30% (Ketley, 1997).

The nucleotide sequence of the genome of *C. jejuni* NCTC11168 has recently been determined (Parkhill *et al.,* 2000). It has been found to be 1,641,481 bp in length and

94.3% of the genome codes for proteins, making *C. jejuni* the most dense bacterial genome sequenced to date (Parkhill *et al.*, 2000). The sequenced genome of NCTC11168 shows a lack of repeated sequences and there is no evidence of functional insertion sequence elements, transposons, retrons or prophages. Excluding clusters involved in lipopolysaccharide (LPS) biosynthesis, extracellular polysaccharide biosynthesis and flagellar modification, there is little organisation of genes into operons or clusters (Parkhill *et al.*, 2000). The genome is also characterised by the presence of hypervariable sequences which are commonly found in genes encoding for the biosynthesis or modification of surface structures (Parkhill *et al.*, 2000).

1.2 Virulence factors and determinants

Bacterial pathogenicity is a multifactorial process. Despite the clinical importance of *C. jejuni*, there is a poor understanding of the virulence determinants of this organism (Parkhill *et al.* 2000). Factors that are generally important in the bacterial disease process include adherance to host tissue, toxin production, colonisation, and host immune system avoidance. A few of these factors are discussed below with regards to *C. jejuni.*

1.2.1 Motility

Motility is required for successful colonisation of the intestinal tract and is therefore considered a major virulence determinant (Ketley, 1997). The flagellum has been the most intensively investigated virulence determinant of *C. jejuni* (Wooldridge & Ketley, 1997). The unique characteristics of the flagellum and the spiral shape of the cell confer a distinctive motility. This may allow *Campylobacter* to colonise the mucous lining of the small intestine (Wooldridge & Ketley, 1997). Szymanski *et al.* (1995) showed that *C. jejuni* exhibited the most efficient swimming behaviour when the viscosity of tissue culture growth medium mimicked the viscosity of the intestinal tract.

The flagella of *C. jejuni* are complex structures composed of two highly related flagellin subunits. Flagellin proteins are the products of two flagellin genes, *flaA* and *flaB*. These genes are highly homologous to each other and are under the control of two distinct and independent promoters (Guerry, 1994). Variations in growth temperature, pH, and inorganic ion contents, leading to increased expression of *flaB,* suggest that *Campylobacter* can modulate the compositions of their flagellin filaments and motilities in direct response to environmental signals (Guerry, 1994). The role of variable expression of flagella in the pathogenesis of *C. jejuni*-associated illness remains to be elucidated (Wallis, 1994) but the association of the flagellin genes with hypervariable regions of the genome suggest a role in the organism's ability to colonise the intestinal tracts of a wide variety of hosts (Lüneberg *et al.,* 1998; Parkhill *et al.*, 2000). Other components of the flagellar machinery, such as the flagellar hook protein, also have roles in the motility and antigenic variability of *C. jejuni* (Kinsella *et al.,* 1997; Lüneberg *et al.,* 1998).

1.2.2 Toxins

1.2.2.1 Exotoxins

Some *Campylobacter* have been described as producing a cholera-like enterotoxin in certain strains, but other investigators have been unable to reproduce these results and evidence for a role and production of an enterotoxin remains unconvincing (Allos & Blaser, 1995; Ketley, 1997; Wassenaar, 1997). A shiga-like toxin has been reported in a single study of some strains of *C. jejuni* and *C. coli,* based on immunological cross-reactivity (Moore *et al.,* 1988, cited by Wassenaar, 1997). However, the evidence remains anecdotal as shiga-like toxic activity has not been reported since the study by Moore *et al.* and there is a lack of homology between the genomes of cytotoxic strains of *C. jejuni* and stx_1 of *E. coli* (Wassenaar, 1997). It has been suggested that some strains produce a hepotoxin, giving the potential to colonise the liver and cause hepatitis, but its nature and mechanism is yet to be elucidated (Wassenaar, 1997).

Cytolethal distending toxin (CDT) has been described in some strains of *C. jejuni* and cloning and sequencing of the structural genes for CDT (*cdtA, B and C*) has facilitated their study (Pickett *et al.,* 1996; Wassenaar, 1997; Eyigor *et al.,* 1999). Pickett *et al.* (1996) reported CDT production in all *C. jejuni* strains tested, but amounts of toxin produced varied. Two of twenty isolates exhibited significantly reduced production and neither were isolated from a healthy human with diarrhoeal disease. One was isolated from a cow and the other was isolated from the spinal fluid of a hypogammaglobulinemic individual. Eyigor *et al.* (1999) suggested *cdt* genes are relatively conserved within species boundaries. The sequence from NCTC11168 showed no evidence of a cholera-like toxin gene, but CDT-encoding genes were present (Parkhill *et al.* 2000).

1.2.2.2 LPS

Lipopolysaccharide (LPS) is a virulence determinant in many enteric pathogens. This endotoxin of Gram-negative bacteria contributes to serum resistance, resistance to phagocytic killing and toxicity (Ketley, 1997). The LPS elicits a host immune response, often resulting in fever symptoms and may also result in irreversible shock and death (Rietschel & Brade, 1992). The lipid A moiety plays a crucial role with regards to the endotoxic properties of the LPS. As a result of molecular mimicry, the core of some LPS molecules of *C. jejuni* may elicit an autoimmune-mediated response against host nerve tissue, resulting in disease manifestations such as Guillain-Barré Syndrome and Miller Fisher Syndrome (Nachamkin *et al.,* 1998; Prendergast *et al.,* 1999). Phase variation of surface antigens is a common mechanism for immune system evasion and can occur by slipped-strand mispairing during replication (Parkhill *et al.,* 2000). The genes responsible for LPS biosynthesis in *C. jejuni* NCTC11168 are coincident with a hypervariable region of the genome, which implies that rapid phase variation can occur (Parkhill *et al.,* 2000).

1.2.3 Adhesion and invasion

Several candidates have emerged as putative *Campylobacter* adhesins but the precise functions and contributions of these to adhesion is not clear (Ketley, 1997). The outer membrane protein PEB1 has been reported to have a role in adherance to host cells (De Melo & Peche're, 1990). Although this gene appears conserved in *C. jejuni* and *C. coli*, *in vivo* evidence of the protein function is lacking (Burnes *et al.*, 1995; Garvis *et al.*, 1996). Flagella may have a secondary role in adherence, enabling attachment to epithelial cell surfaces (McSweegan & Walker, 1986). However, this role is not supported elsewhere (Ketley, 1995) and there is little direct evidence to separate the involvement of flagellar components and motility (Ketley, 1997). The short O side chains of *C. jejuni* LPS molecules have been implicated in adhesion to INT407 epithelial cells (McSweegan & Walker, 1986). Periodate oxidation of the LPS was shown to reduce LPS binding to cells, suggesting binding is via the carbohydrate portion (McSweegan & Walker, 1986). Doig *et al.* (1996) demonstrated the production of an environmentally regulated pilus-like appendage in *C. jejuni, C. coli* and *C. fetus*, in response to the presence of bile salts. The precise role of the putative pilus in the disease process remains unknown. A non-pilated isogenic mutant of *C. jejuni* 81-176 was still able to adhere to and invade INT407 cells and colonise ferrets, but with ameliorated disease symptoms (Doig *et al.*, 1996). The protein CadF (*Campylobacter* adhesin to fibronectin) has recently been identified as a *Campylobacter* adhesin (Konkel *et al.* 1997). The CadF protein appears conserved among *C. jejuni* and *C. coli* isolates, as indicated by immunoblot analysis (Konkel *et al.* 1997; Konkel *et al.* 1999b).

Bacterial internalisation has typically been observed to involve rearrangement of the host cytoskeleton structure, resulting in endocytosis of the pathogen (Hu & Kopecko, 1999). Hu and Kopecko (1999) provided direct evidence for the involvement of microtubules and dynein in *C. jejuni* internalisation, by time-course immunofluorescence microscopic analyses of uptake of *C. jejuni* 81-176 in INT407 cells, has been provided (Hu & Kopecko, 1999). Recently, the *ciaB* gene has been identified, encoding a protein required for the internalisation of *C. jejuni* by non-professional phagocytic cells (Konkel *et al.* 1999b). This was demonstrated by Konkel *et al.* (1999a) as a significant reduction in the internalisation of *ciaB* mutants when compared to the parental isolate.

1.2.4 Iron acquisition

Iron acquisition is a crucial aspect of bacterial infectivity and this system is likely to be important in virulence (Gonzalez *et al.*, 1997). *Campylobacter* spp. are not thought to produce siderophores but are able to utilise ferrichrome and enterochelin as sources of iron (Richardson & Park, 1995). A transport system, encoded by the *ceu* operon, that confers the ability to utilise enterochelin has been described for both *C. jejuni* and *C. coli* (Richardson & Park, 1995; Gonzalez *et al.*, 1997). The iron storage protein ferritin, encoded by the *cft* gene, is produced by *C. jejuni* and may facilitate host colonisation and provide protection against high O_2 levels (Wai *et al.*, 1995, cited by Ketley, 1997). In Gram-negative bacteria the Fur protein regulates gene expression in response to intracellular iron concentrations (van Vliet *et al.*, 1998). A *C. jejuni fur* mutant exhibited lower growth rates than that of the wild-type isolate under both low- and high-iron conditions (van Vliet *et al.*, 1998). AhpC is an iron-repressed protein and has been shown to confer aerotolerance and oxidative stress resistance in *C. jejuni* (Baillon *et al.*, 1999). However, it is not under Fur regulation, suggesting the presence of Fur-independent iron regulation (van Vliet *et al.*, 1998).

1.2.5 Involvement of plasmids

Plasmids have been found in between 19 and 53% of *C. jejuni* strains and are generally reported to be R plasmids (Bacon *et al.*, 2000). However, partial sequence analysis of one plasmid, termed pVir, of *C. jejuni* 81-176 revealed open reading frames (ORFs) encoding predicted proteins with strong similarity to *Helicobacter pylori* proteins. One ORF had strong similarity to a protein encoded by the *cag* pathogenicity island (Bacon *et al.*, 2000). ORF1 through ORF3 encoded proteins which displayed significant identity to the products of *H. pylori* genes *comB1*, *comB2* and *comB3*, involved in natural transformation and DNA uptake. ORF4 had significant identity to VirB11, proposed to function in a type IV secretion system required for virulence (Bacon *et al.*, 2000). Site-specific insertional 81-176 mutants of *comB3* and *virB11* exhibited significant reduction in adherence and invasion

of INT407 cells and reduced virulence in a ferret diarrhoeal disease model. Although the ferret model may be one of the more useful models (Fox, 1992), animal models may not be an accurate representation of pathogenesis in humans and may be difficult to interpret (Black *et al.*, 1988). The *comB3* mutants showed decreased natural transformation efficiency but the role of *comB3* in adhesion and invasion was not investigated (Bacon *et al.*, 2000). Bacon *et al.* (2000) suggested their data indicated different mechanisms by which different strains of *C. jejuni* may cause disease. This was supported by their observation that only 10% of fresh clinical isolates probed for *virB11* were positive for this gene.

1.3 Pathogenic potential of *C. jejuni*

The question of whether all strains of *C. jejuni* are equally pathogenic to humans has long been debated. Like other established enteropathogens, *C. jejuni* can cause diarrhoea in susceptible children and adults but can also cause asymptomatic infections, especially in children in developing countries exposed from an early age (Black *et al.*, 1988). Some strains appear more invasive than others. For instance, strain 81-176 exhibits highly efficient internalisation of epithelial cell lines *in vitro* (Hu & Kopecko, 1999) and has demonstrated a high virulence level in contrast to strain A3249 in human volunteer studies (Black *et al.*, 1988).

1.3.1 Volunteer studies

Volunteer studies lend support to the hypothesis that strains of *C. jejuni* do differ in pathogenicity (Black *et al.*, 1988). One volunteer study showed illness was more severe in participants challenged with strain 81-176 than in those challenged with strain A3249 (Black *et al.*, 1988). On the basis of higher attack rates at equal doses and a greater number and volume of stools produced, the strain 81-176 appeared to be more pathogenic than strain A32249 (Black *et al.*, 1988). 81-176 was isolated from an outbreak and the symptoms exhibited by the people affected in the outbreak were more severe than the symptoms of the sporadic infection by A3249 (Black *et al.*, 1988).

1.3.2 Strain invasiveness

Blaser *et al.* (1986) investigated whether isolates from gastroenteritis patients were more susceptible to normal human serum than isolates from patients with extraintestinal disease. The distribution of serum susceptibilities of the faecal isolates was nearly identical to that of the blood isolates (Blaser *et al.* 1986). They suggested extraintestinal spread of *C. jejuni* should be considered opportunistic and for extraintestinal infection to occur in a normal host, increased virulence must be present. The opportunistic nature of *C. jejuni* is exemplified by recent fatal bacteraemia cases in AIDS patients (Manfredi *et al.,* 1999). Interestingly, there was an absence of enteric infection in these cases. Clearly, the influence of host factors on disease outcome may be significant, but that some strains may be more virulent than others cannot be ruled out.

A study by Carvalho *et al.* (2001), using RAPD fingerprints to distinguish invasive *Campylobacter* strains from noninvasive strains, observed an 1.6 kb fragment more frequently associated with invasive strains. Specific PCR amplification of this *iam* (invasion-associated marker) locus revealed its presence in 85% of invasive strains compared with 20% of non-invasive strains. The PCR-generation of false-positives and false-negatives did occur at rates of 25% and 14%, respectively, but it was not indicated how this was tested. An *iam*-specific DNA probe only hybridised to the 1.6 kb fragment in the RAPD fingerprints. However, the whole genome was not probed, and therefore genomic rearrangements may have led to the locus to be present elsewhere in the genome and may not have been detected by RAPD-PCR. No sequence or mutagenesis analysis was performed to ascertain the relative invasive potential of strains carrying the *iam* locus. However, the study indicates carriage of the *iam* locus may influence disease outcome. A similar study demonstrated an 1.4 kb fragment with strong homology to *gyrB* of *H. pylori*, present in HS:19 strains but absent from non-HS:19 strains (Misawa *et al.,* 1998). Nothing was inferred from this study as to the potential of HS:19 strains to cause GBS, other than HS:19 strains appear to be a highly clonal, and perhaps a more virulent, population. This finding of clonal virulent populations is consistent with what is observed in *N. meningitidis* isolates (Holmes *et al.,* 1999).

1.3.2.1 Guillain-Barré Syndrome: An example of strain-specific pathogenesis?

C. jejuni is the most frequent infectious agent associated with the development of GBS (up to 66% of GBS patients have had a recent *C. jejuni* infection) (Prendergast *et al.*, 1999), indicating *C. jejuni* is an important causal factor for GBS (McCarthy & Giesecke, 2001). GBS is an autoimmune disorder of theperipheral nervous system characterised by by ascending paralysis that can lead to respiratory muscle compromise and death (Misawa *et al.*, 1998; Nachamkin *et al.*, 1998). The risk of developing GBS after a *C. jejuni* infection is estimated at approximately 1 in 1 000 but may be higher depending on the strain involved (Nachamkin *et al.*, 1998; Endtz *et al.*, 2000).

Some Penner serotypes, particularly HS:19 (O:19) and HS:41 (O:41), are more frequently reported with the incidence of GBS and Miller Fisher Syndrome (MFS), a rare variant of GBS (Nachamkin *et al.*, 1998; Prendergast *et al.*, 1999; McCarthy & Giesecke, 2001). A study in Japan by Yuki *et al.* (1997) reported 52% of *C. jejuni* strains isolated from GBS patients were serotype HS:19, compared with 5% of strains from patients with uncomplicated gastroenteritis (cited by Nachamkin *et al.*, 1998). One study found five of nine GBS-related strains, isolated from diverse countries, were HS:19 and these five strains were clonally related, based on *flaA*-RFLP and RAPD analysis (Fujimoto *et al.*, 1997, cited by Endtz *et al.*, 2000). HS:41 strains have been highly associated with GBS in South Africa and also occurs frequently in uncomplicated infections (Nachamkin *et al.*, 1998). In South Africa, HS:41 strains were rarely isolated from uncomplicated infections, suggesting a possible emergence of this serotype in association with GBS in that geographic region (Goddard *et al.*, 1997). HS:2 strains were over-represented compared to control isolates in a study of isolates of MFS patients (Nachamkin *et al.*, 1998). In contradiction, a study of *C. jejuni* isolates from GBS and MFS patients in the Netherlands did not demonstrate over-representation of specific HS serotypes (Endtz *et al.*, 2000). Genotypic techniques also showed substantial genetic heterogeneity in GBS- or MFS-related strains, with no clustering of GBS- or MFS-related strains in the Dutch study.

As stated previously, it is hypothesised that some LPS species of *C. jejuni* elicit an autoimmune-mediated response against host nerve tissue (Nachamkin *et al.,* 1998; Prendergast *et al.,* 1999). Autoreactive antibodies to gangliosides, especially GM_1, are found in 20% of GBS patient sera after *C. jejuni* infection. In *C. jejuni* HS:19 strains, the terminal regions of the LPS core have been shown to have structural identity with the human gangliosides GM_1, GD_{1a}, GT_{1a} and GD_3 (Aspinall *et al.,* 1994). Mimicry of gangliosides is not limited to strains associated with GBS, which suggests host or other bacterial factors are involved in disease pathogenesis (Prendergast *et al.,* 1999).

1.3.3 Source overlap

Studies have shown there is not a complete overlap of strains isolated from one source (eg., poultry) and strains isolated from humans (Kakoyiannis *et al.,* 1988; Koenraad *et al.,* 1995; Korolik *et al.,* 1995; Hudson *et al.,* 1999). These studies suggest not all strains cause disease in humans. They also indicate a diverse range of reservoirs of strains that are potential sources of *Campylobacter* infection, as some human isolates have been indistinguishable from isolates from different animal and environmental sources (Hudson *et al.* 1999). Raw or undercooked poultry has been identified as a major source of sporadic campylobacteriosis in humans. Poultry flock colonisation is often restricted to one strain and the predominance of certain subtypes that are relatively absent in human infections suggests a lower level of virulence (Koenraad *et al.,* 1995; Wassenaar *et al.,* 1998).

Korolik *et al.* (1995) suggested that only a small proportion of *C. jejuni* strains found in chicken flocks are capable of causing disease in humans. This conclusion was based on probe hybridisation to different sized fragments in the respective *Cla*I restriction enzyme profiles of isolated DNA from strains isolated from chickens and humans. The probe used in the Southern hybridisations, pMO2005, encoded a *C. jejuni* membrane antigen. The *C. jejuni* strains could be divided into two groups on the basis of this differential probe hybridisation. Korolik *et al.* reported an overlap of approximately 30% of strains from

each source. This cannot be presented as irrefutable evidence of differences in pathogenic potential of strains from different sources, as only a small number of isolates from each source were analysed and similar polymorphism was not observed in *Eco*RV restriction profiles. It must also be noted that in this study chicken and human strains were indistinguishable based on restriction profiles alone. However, the study does indicate there is not a complete overlap between strains from poultry and those from humans which may suggest a lower pathogenic potential in some chicken strains. Another study, using bacterial restriction endonuclease DNA analysis (BRENDA) confirmed poultry as a major source of *C. jejuni* in humans (Kakoyiannis *et al.*, 1988). However, only 35% of BRENDA profiles for human isolates were indistinguishable from approximately 50% of poultry isolates. Recent multi-locus sequence typing (MLST) data suggested that it may be unlikely that the majority of the human strains come from chickens (Dingle *et al.*, 2001). However, only a small number of strains have been analysed by MLST and therefore it is difficult to judge the significance of these findings.

A New Zealand study has compared pulsed-field gel electrophoresis (PFGE) types and Penner serotypes of *C. jejuni* isolated from humans and other animal and environmental sources (Hudson *et al.*, 1999). It was reported that amongst isolates indistinguishable by both typing methods and represented by more than one strain, there were types from other sources that were not isolated in humans (Hudson *et al.*, 1999). In this study, there were also types that were only identified from human isolates. This data may be interpreted to suggest that although *Campylobacter* is ubiquitous in the environment, not every strain may pose a risk to human health. However, interpretation of this data in terms of pathogenic potential is difficult, due to the limited number of isolates used in this study. The study by Hudson *et al.* also highlights there are potentially many unrecognised sources of *C. jejuni* infection.

In contrast, Aeschbacher and Piffaretti (1989) concluded that, based on multi-locus enzyme electrophoresis (MLEE), human and animal strains do not constitute subpopulations and every animal strain may be considered a potential human pathogen. This has been supported

by a study using amplified fragment length poymorphism (AFLP), where clusters containing both human and poultry isolates were reported (Duim *et al.*, 1999). No source-specific clusters were observed. MLEE uses variation that is selectively neutral (ie., changes in the amino acid composition of protiens) and accumulating slowly in the population to discriminate between strains (Maiden *et al.*, 1998).

The studies mentioned in this section present two distinguishable lines of evidence regarding the pathogenic potential of *C. jejuni*. The variation between strains is evolving rapidly, as judged by techniques such as PFGE and RAPD-PCR (Maiden *et al.*, 1998). This may explain the discrepancy between Aeschbacher and Piffaretti (1989), and other studies, but would not explain the observations of Duim *et al.* (1999). A subset of poultry strains found to cause disease in humans may have evolved this characteristic relatively recently. This recent change may not be evident with MLEE (eg., it has arisen by horizontal gene transfer). However, a study using MLST, a technique based on the same principles as MLEE but more discriminatory (ie., every base is a marker), clearly indicated a lack of overlap between poultry and human isolates (Dingle, *et al.*, 2001). There is mounting evidence to suggest that strains not only vary in virulence but mechanisms of disease may also vary between strains (Bacon *et al.* 2000). Therefore, despite contradictory lines of evidence, the pathogenic potential of *C. jejuni* strains may indeed vary and warrants further investigation.

1.3.4 Colonisation potential

It has been observed that different isolates of *C. jejuni* exhibit different potentials for caecal colonisation of chicks (Stern *et al.*, 1988, cited by Payne *et al.*, 1999). This is supported by a RAPD-PCR study that suggested varying colonising potentials for isolates with different RAPD profiles (Payne *et al.*, 1999). A novel two-component regulatory system (TCR), designated the RacR-RacS system, has been described for *C. jejuni* (Brás *et al.*, 1999). Mutation of the response regulator gene, *racR*, conferred a reduced growth rate at 42°C and an inability to achieve parental cell density level in tissue culture and the

mutant exhibited reduced colonisation efficiency in chickens. It is reasonable to assume that a strain that is efficient in colonising poultry will not necessarily be efficient in causing disease in humans. Different host-specific virulence determinants may be necessary in order to efficiently colonise and manifest disease in humans.

1.4 Selection pressures

Slipped-strand mispairing, as well as recombination or large-scale genomic rearrangements, may be useful in the adaptation of an organism for colonisation and survival in the gut of a variety of hosts (Parkhill *et al.,* 2000; Hänninen *et al.* 2001). The predominance of certain strains in poultry may reflect enhanced survival of such strains in different environments (Wassenaar *et al.,* 1998). A recent MLST study observed little sequence diversity in the *C. jejuni* isolates studied, most of which were isolated from humans (Suerbaum *et al.,* 2001). It was suggested that this was a consequence of the rapid expansion of the *C. jejuni* population, driven by changes in food animal husbandry and slaughtering practices in the last one or two centuries (Suerbaum *et al.,* 2001). Modern animal husbandry practices provide a large pool of genetically interconnected bacteria where indiscriminate use of antibacterial agents aids selection for rare recombinational events (Gibreel *et al.,* 1998). Integrons, a class of mobile genetic elements, may also facilitate the spread of antimicrobial resistance in strains from animal sources and clinical isolates (Lucey *et al.,* 2000). However, integrons were not identified in *C. jejuni* NCTC11168 (Parkhill *et al.,* 2000).

The selection pressure provided by the use of antimicrobials in animal husbandry is evident by the rapid emergence of resistant strains of *C. jejuni.* Although trimethroprim resistance is considered endogenous in *C. jejuni,* the trimethroprim resistance genes, *dfr1* and *dfr9* were found to occur simultaneously in only 10% of strains. Where both genes were absent resistance levels were found to be lower than other strains (Gibreel *et al.,* 1998). Prior to this 1998 study, the *dfr9* gene had previously only been reported in porcine isolates of *E. coli.* One third of clinical *C. jejuni* isolates from Sweden, where trimethroprim is used

extensively in swine rearing, contained *dfr9* (Gibreel *et al.* 1998). After the approval of fluoroquinolone use in poultry in both the US and Europe (1995), emergence of a large population of resistant *C. jejuni* strains in humans appeared (Altekruse *et al.,* 1999). Ciprofloxacin resistance in *Campylobacter* strains from broilers in Spain in 1997-1998 was reported to be 99%, where no resistance had been reported prior to 1988 (Sáenz *et al.,* 2000).

It has been suggested that animal husbandry and animal-processing units may facilitate *Campylobacter* contamination of associated water systems (Buswell *et al.*, 1998). Under certain conditions, particularly low temperatures and association with biofilms, *Campylobacter* spp. can survive for a considerable length of time. Strain variation in survival could contribute to certain strains being of particular concern for human and animal infection (Buswell, *et al.*, 1998). Certain stressful environmental conditions, such as oxygen exposure and nutrient limitation may trigger a transition from rod to coccoid shape, associated with a viable non-culturable (VNC) state (Ketley, 1997). It is suggested the VNC state is an adaptation to survival in adverse environments, although the evidence for this is contradictory (Ketley, 1997).

1.5 A non-clonal population structure?

The shotgun assembly library used for sequencing NCTC11168 revealed regions in which the sequences of otherwise identical clones varied at a single point (Parkhill *et al.,* 2000). The high level of variation observed was not consistent with a shotgun library derived from a clonal population and means that it is not possible to produce a definitive sequence for the *C. jejuni* genome. Based on MLST *C. jejuni* has been reported to have a nucleotide sequence diversity similar to that of *Neisseria meningitidis*, which is relatively non-clonal (Dingle *et al.,* 2001).

Macrorestriction profiling has indicated that some groups represent genetic lineages among the highly diverse genotypes of *C. jejuni* and these genotypes seem to persist from one

year to another (Hänninen *et al.*, 2001). However, there may be changes within these genotypes that are not reflected within the macrorestriction profiles generated. MLEE analysis has suggested a heterogenic population structure for *C. jejuni* (Aeschbacher & Piffaretti, 1989). Hänninen *et al.* (2001) suggested certain strains with shared genotypes and phenotypes may become locally predominant and form temporary clonal groupings. These clonal groupings may be due to specific characteristics that are advantageous for their colonisation in animals or for their environmental transmission and pathogenicity in humans. This is consistent with what is observed in *N. meningitidis,* where recombination is common (Maynard Smith *et al.*, 1993; Holmes *et al.*, 1999).

Data from a MLST study showed that populations of *C. jejuni* are characterised by a low degree of sequence diversity, a relatively small pool of alleles in the housekeeping genes tested, and high rates of intra-species recombination which is frequent enough to generate a large number of unique combinations of sequence types (Suerbaum *et al.*, 2001). The recombinational nature of *C. jejuni* would indicate a largely non-clonal population (Maynard Smith *et al.*, 1993), yet clonal groupings of relatively recent evolutionary origin can emerge (Dingle *et al.*, 2001; Hänninen *et al.*, 2001).

1.6 Genomic instability of *C. jejuni*

The genome of *C. jejuni* is extremely heterogenous, as judged by molecular typing techniques (Hänninen *et al.*, 2001; Suerbaum *et al.*, 2001). This heterogeneity may result from genomic instability (Hänninen *et al.*, 1999). Two different PFGE profiles have been reported for the strain NCTC11168 which may reflect the instability of the genome, indicate different data interpretations, or indicate different experimental conditions (Karlyshev *et al.*, 1998). Genome instability is supported by observations from the sequencing of this strain (Parkhill *et al.*, 2000). PFGE profiles of some *C. jejuni* strains have been shown to change after passage through a chick intestine (Hänninen *et al.*, 1999). Another PFGE study suggested isolates from meat processing batches were of clonal origin but had

undergone genomic rearrangements, however, instability could not be confirmed upon *in vivo* passage (Wassenaar *et al.,* 1998). Similar observations of genetic instability have also been made for *C. coli*, where PFGE profiles of some *C. coli* strains changed significantly after extended subculture under standard *in vitro* culture conditions (On, 1998). Studies have shown that some *C. jejuni* and most *C. coli* strains are naturally competent during the logarithmic phase of growth and that these strains show strong selectivity for uptake of DNA from their own species (Wang & Taylor, 1990). However, *C. coli* has been demonstrated to chromosomally integrate plasmid DNA at random sites, with little or no homology to the *C. coli* chromosome (Richardson & Park, 1997).

A study using BRENDA reported these profiles as very stable, even after numerous *in vitro* passages and over a period of months (Kokoyiannis *et al.,* 1988). Stability of profiles after *in vivo* passage was not reported. Contrary to Hanninen *et al.* (1999) it was suggested that minor differences in profile may represent a unique BRENDA type and not recent DNA mutations (Kokoyiannis *et al.*, 1988). Although BRENDA and PFGE are different techniques, the different interpretations of minor differences in restriction fragment profiles make judgements of strain relatedness difficult. The criteria suggested by Tenover *et al.* (1995) for the interpretation of PFGE profiles, accounts for random genetic events when determining the relatedness of isolates. On *et al.,* (1998) emphasised the importance of confirming identical profiles by the use of two or more restriction enzymes when performing macrorestriction profiling of nonepidemiologically-linked isolates.

1.7 Recombination in other bacteria

Observations from MLEE experiments suggest the population structure of most studied bacteria is clonal (Maynard Smith *et al.,* 1993). Maynard Smith *et al.,* (1993) explain high coefficients of linkage disequilibrium, usually indicating clonal population structure, can arise in several ways in bacterial populations in which recombination is frequent. Therefore, the widespread occurrence of a single electrophoretic type (ET) cannot be taken as evidence

of clonality if the number of isolates with that ET is similar to that which would be expected if loci are randomly assorting (Maynard Smith *et al.,* 1993). The study by Maynard Smith *et al.* (1993), applying MLEE to investigate population structure, showed that bacterial population structure ranges from effectively panmictic (eg., *Neisseria gonorrhoeae*) to one that is clonal on all levels (eg., *Salmonella* spp.).

1.7.1 *Neiserria meningitidis*

The way in which phylogenic data is presented may not accurately represent the population structure for that organism (Maynard Smith *et al.,* 1993; Holmes *et al.,* 1999). Holmes *et al.* (1999) addressed this problem in *N. meningitidis* by asking if its population structure is best represented by a bifurcating tree as predicted by a clonal model (ie., no recombination occurs between isolates of the same, or different, branches of the tree), as a network of interconnected nodes expected with frequent recombination, or as a star phylogeny in which lineages have arisen within a short time period. The observations of Holmes *et al.* (1999), based on MLST data, were consistent with the hypothesis that meningococcal populations comprise organisms assembled from a common gene pool, with alleles and allele fragments spreading independently, with occasional importation of genetic material from other species. They concluded that recombination within meningococcal populations is sufficient to disrupt a branching tree-like phylogeny and this would not be an appropriate model for the long-term evolution of *N. meningitidis.*

Organisms such as *N. meningitidis* present a particular challenge to molecular typing because the extent of recombination is higher than in most bacterial populations (Maiden *et al.,* 1998). Most invasive meningococcal disease in the developed world has been associated with a small number of what are termed hyper-virulent lineages. Due to the high frequency of recombination relative to the mutation rate, hyper-virulent lineages are expected to emerge at intervals within a population. Highly localised recombinational events slowly diversify initially uniform genomes to an extent where they are no longer distinguishable from the background population (Maiden *et al.,* 1998).

1.7.2 *Helicobacter pylori*

Helicobacter pylori, closely related to *Campylobacter,* was reportedly the first organism to have the complete genomic sequences of two unrelated strains, J99 and 26695, compared (Alm *et al.,* 1999). J99 was isolated from a patient with a duodenal ulcer and 26695 was from a gastritis patient. This comparison essentially provided a random sampling of species variation (Lan & Reeves *et al.,* 2000). Although *H. pylori* is believed to exhibit a large degree of genomic and allelic diversity, Alm *et al.* (1999) reported the overall genomic organisation, gene order and predicted proteomes were quite similar. However, 6% to 7% of the genes were specific to each strain, with almost half of these genes being clustered in a single hypervariable region (Alm *et al.,* 1999). Hypervariable regions have also been reported for *C. jejuni* (Parkhill *et al.*, 2000). Strain-specific genes, such as those associated with the hypervariable region, may play a role in the pathophysiology and severity of *H. pylori* infection, and differential gene expression, perhaps mediated by slipped-strand repair, may affect the ability of the organism to colonise (Alm *et al.*, 1999). Alm *et al.* (1999) suggested that results obtained with lower-resolution techniques, such as PFGE and PCR-RFLP have probably led to an overestimation of genetic diversity in *H. pylori* and the absence of extensive gene shuffling in the respective strains is consistent with a low level of evolutionary divergence. Strain-specific DNA-restriction/modification genes with a lower G+C content were associated with regions organised differently in the respective strains. Alm *et al.* (1999) inferred these genes may have been acquired horizontally from other bacterial species or transferred more recently from other *H. pylori* strains by natural transformation.

H. pylori strains are grouped into two broad families termed type I and type II, associated with severe disease pathology and attenuated virulence respectively. Type I strains have been found to possess a 37-40 kb insertion called the *cag* pathogenicity island (PAI), which carries the *cagA* gene (Censini *et al.* 1996; Akopyants *et al.*, 1998). The *cag* PAI has a reported G+C abundance of 35%, compared to 38-45% reported for *H. pylori* genes and the presence of direct repeats flanking the *cag* PAI suggest it has been acquired by a recombinational event (Censini *et al.*, 1996). Censini *et al.* (1996) suggested these

observations indicated the *cag* PAI may be derived from a plasmid or phage via horizontal transmission. That the structure of the *cag* PAI was not identical in all type I strains indicated that the region has gone through a series of rearrangements in different strains, which may play a significant role in the pathogenic evolution of *H. pylori* (Censini *et al.*, 1996). One study determining the prevalence of *cagA* found there was a higher, but statistically insignificant, percentage of *cagA*-negative strains in a non-ulcer group compared with an ulcer group (Ryan *et al.*, 2001). Although consistent with previous observations that *cagA* predominates in patients with more severe pathologies, it suggested *cagA* is not a predictor of disease outcome in some human populations (Ryan *et al.*, 2001).

1.7.3 Pathogenicity islands

The existence of pathogenicity islands (PAIs) in *C. jejuni* has not been reported. However their acquisition in other pathogenic bacteria has been important in the evolution of pathogenesis. PAIs in other bacteria carry genes that confer aspects of pathogenicity (Lan & Reeves, 2000). This kind of intra-species variation is of great consideration when studying pathogenicity (Lan & Reeves, 2000).

Dichelobacter nodosus differ in virulence potency from strain to strain and the degree of virulence corresponds with the presence of multiple copies of the *vap* region (Hacker *et al,*. 1997). However, the fully sequenced *C. jejuni* NCTC11168 was found to have an unusually low number of repetitive sequences (Parkhill *et al.*, 2000) so the likelihood of a similar mechanism in NCTC11168 would be low. This statement may not be representative of all *C. jejuni* strains. The *vap* gene products of *D. nodosus* show similarities to plasmid encoded proteins of other Gram-negative bacteria, and a plasmid of *D. nodosus* representing a circular form of the *vap* region has also been described, indicating PAI formation occurred by integration into the chromosome (Hacker *et al.*, 1997).

Serovars of *Salmonella enterica* are differentiated on the basis of PAI acquisition. *Salmonella* spp. represent an extremely clonal population and acquisition of specific PAI is thought to

have played a significant role in the evolution of this pathogen (Maynard Smith *et al.*, 1993; Bäumler, 1997; Lan & Reeves, 2000). SPI-1 governs the ability of salmonellae to enter mammalian epithelial cells while SPI-2 is required for survival within macrophages. Due to the critical role of these PAI in *Salmonella* pathogenesis, both SPI-1 and SPI-2 are stable within the population (Ochman & Groisman, 1996). Similarly, specific pathogenicities of *E. coli* strains are linked to the acquisition of specific PAIs. However, unlike *Salmonella* spp., pathogenic *E. coli* possess distinct mechanisms for causing disease (Bäumler, 1997).

Natural genetic competence and high recombination efficiencies are associated with PAI acquisition (Hacker *et al.* 1997). *C. jejuni* possesses both of these traits (Wang & Taylor, 1990; Suerbaum *et al.*, 2001). PAI acquisition is also known in the closely related species *H. pylori*. This might suggest a good likelihood of PAI acquisition in *C. jejuni*. *C. jejuni* also has a propensity for survival in a wide variety of hosts. It has been suggested that the capacity of *Yersinia* spp. to lose and regain PAIs may represent a strategy for adaptation to different environments (Hacker *et al.*, 1997).

1.8 Traditional techniques for studying virulence in *Campylobacter*

Until the development of a Tn*553*-based transposon mutagenesis technique (Colegio *et al.*, 2001), mutagenesis in *C. jejuni* had been inefficient and alternative methods for the elucidation of genes with potential importance for pathogenicity have been required. Several methods have been developed to facilitate the understanding of genetic determinants of *Campylobacter* virulence.

Shuttle mutagenesis is one approach that has been used to study potential virulence genes (Labigne-Roussel *et al.*, 1988). This approach is often used in conjunction with suicide vectors which can be introduced into *Campylobacter* but cannot replicate (Labigne-Roussel *et al.*, 1988; Taylor, 1992). A resistance-cassette is inserted into the cloned gene of interest

on a suicide vector and incorporated into the chromosome by homologous recombination (Labigne-Roussel *et al.*, 1988). In this way precise insertional mutations in a gene of interest are possible. The effect of gene disruption may then be assayed and gene function elucidated. This approach has been used successfully in a number of studies (Yao *et al.*, 1994; Doig *et al.*, 1996; Konkel *et al.*, 1997; Konkel *et al.*, 1999). However, shuttle mutagenesis only allows insertional disruption of a specific gene, and the gene of interest must already be cloned (Taylor, 1992).

An alternative method of insertional mutagenesis using natural transformation has been developed (Bleumink-Pluym *et al.*, 1999). In the demonstration of this method, a chromosomal library was made in the shuttle vector pUOA18 and a kanamycin-resistance cassette was ligated into the inserts of the plasmids. *C. jejuni* 81-116 was transformed with the modified plasmid and the chromosomal DNA of the resulting transformants was used to retransform *C. jejuni* 81-116. The mutants were screened for motility and eleven of a total of 1 300 Km^R transformants were found to be non-motile. The results obtained from the study suggested the mutants generated by this technique were not completely random (Bleumink-Pluym *et al.*, 1999).

An *in vivo mariner*-based transposon mutagenesis system for the production of random insertional mutants of *C. jejuni* has been tested and has demonstrated that the mini-transposon, *Himar1*, inserts with a high degree of randomness throughout the *C. jejuni* chromosome (Golden *et al.*, 2000). A transposon mutagenesis system based on the *Staphylococcus aureus* transposable element Tn*552* showed more promise as it generated up to ~8 000 random mutants and the technique was deemed more simplistic (Colegio *et al.*, 2001). These recent developments in mutagenesis approaches will greatly facilitate the study of pathogenicity in *C. jejuni.*

1.9 Techniques to detect intra-species variation

There are a number of techniques now available to detect intra-species variation. These techniques fall into one of two categories: variation at the genomic level and variation at the expression level.

A number of typing methods detect intra-species variation. PFGE has been applied widely to *Campylobacter* species. MLST and RAPD-PCR have also been used. Although these methods have been successful in identifying strains for epidemiological studies, they lack application to the study of pathogenicity. As mentioned previously, RAPD-PCR has been applied to identify differences between *C. jejuni* isolates, with respect to pathogenic potential (Misawa *et al.,* 1999; Carvalho *et al.,* 2000). The relationship of these differences to the respective pathogenic potentials of the strains was then investigated. PFGE could similarly be applied but this has not been reported. MLST is a useful epidemiological tool (Dingle *et al.,* 2001), but would not be ideal for adaptation to the study of pathogenic potential. In MLST, the variation designed to be detected is in the house-keeping genes.

One method that has been applied widely to investigate intra-species variation in other bacteria, and shown to be useful in the study of pathogenicity, is subtractive hybridisation. Variation between strains can also be detected at the level of expression. Differential expression of genes can be detected with such methods as mRNA-based subtractive hybridisation, differential-display PCR (DD-PCR) and RNA fingerprinting by arbitrarily-primed PCR (RAP-PCR).

The use of either DNA- or mRNA-based subtractive hybridisation to identify potential virulence determinants in *Campylobacter* species has not been reported in the literature. A technique such as subtractive hybridisation is an attractive candidate for the study of pathogenic potential in *C. jejuni.* It has great potential in the comparison of genomic sequences between related bacterial strains that differ in virulence (Sawada *et al.*, 1999) and is a powerful tool for rapid and directed analysis of the basis of inter- or intraspecifc phenotype variations (Tinsley & Nassif, 1996).

1.10 Subtractive hybridisation

The concept of subtractive hybridisation was pioneered by Bautz and Reilly in 1966, who used DNA from a bacteriophage T4 deletion mutant to isolate mRNAs from the deleted region (cited in Straus & Ausubel, 1990). However, it has not been until more recently that methods for enrichment of deleted DNA sequences from genomic DNA have been developed. Several additional methods have been developed independently (Straus & Ausubel, 1990; Wieland *et al.,* 1990; Bjourson *et al.*, 1992).

Regardless of whether subtractive hybridisation is based on genomic differences (DNA-based) or differences in gene expression (mRNA-based), the basic principles remain the same. The fundamental principle of all subtractive hybridisation protocols is the removal of nucleic acid sequences from one cell type or strain which are homologous to sequences from a different cell type or strain, leaving fragments that can only be found in the strain of interest (Bjourson *et al.,* 1992). In subtractive hybridisation protocols, the strain with the characteristic of interest, eg., virulence, is often termed the "tester". The tester contains target sequences that are not present in the other strain, termed the "driver". When the tester and the driver DNAs or cDNAs are mixed, driver DNA is always provided in excess. This combined pool is subsequently denatured and rehybridised. DNA or cDNA that is present in the driver but absent in the tester is enriched either by physical removal of the tester-driver and driver-driver hybrids or by kinetic enrichment of tester-tester hybrids via PCR.

1.11 Fundamentals of subtractive hybridisation

The fundamental aspects of the subtractive hybridisation technique are discussed in more detail below. These are discussed primarily from the DNA-based perspective but apply similarly to mRNA-based protocols.

1.11.1 Fragmentation of tester and driver DNA

In DNA-based protocols the starting DNA is either digested with restriction endonucleases or mechanically sheared. This process provides some degree of uniformity to the length of DNA fragment. This may not be required in mRNA-based protocols where cDNA is synthesised by random-priming. Depending on the specific application and what organism is involved, *Sau*3A is frequently used to digest tester DNA to an average length of 500 bp (Straus & Ausubel, 1990; Bjourson *et al.*, 1992). The same enzyme may be used to digest the driver DNA (Bjourson *et al.*, 1992; Akopyants, 1998) or it may be sheared by sonication to a length of approximately 1 to 3 kb (Straus & Ausubel, 1990; Brown & Curtiss, 1996). Straus and Ausubel, who applied the technique to isolation of DNA corresponding to a deletion mutant, stressed the deletion must cover at least one restriction site that has been used. However, this appears of little relevance where subtractive hybridisation has been applied more generally to find differences between strains. The size of fragment required to provide optimal recovery of unique sequences may be of greater consideration. Using smaller DNA fragments avoids the removal of potentially unique sequences which may be contained in a large fragment that also contains regions of homology with the subtracter DNA (Bjourson *et al.*, 1992).

1.11.2 Ratio of tester to driver DNA

The mass ratio of tester to driver DNA can vary greatly between protocols. Ratios as low as 1:20 (Straus & Ausubel, 1990; Brown & Curtiss, 1996) and as high as 1:4 000 (Bjourson *et al.*, 1992) have been reported. More commonly ratios between 1:50 and 1:200 are used. The large excess of driver DNA is used to remove sequences common to the tester, thereby enriching target sequences unique to the tester (Wieland *et al.*, 1990). For efficient subtraction, the tester to driver ratio often needs to be optimised (Schmidt *et al.*, 1998). The ratio of tester to driver DNA is considerably lower in mRNA-based protocols. Mass ratios of 1:5 (Utt *et al.*, 1995; McGowan *et al.*, 1998) and 1:10 (Plum & Clark-Curtiss, 1994) appear to be standard.

1.11.3 Hybridisation stringency

The specificity of the nucleic acid sequences to the tester strain generated by the subtraction is dependent on the stringency at which the hybridisation takes place. A high-stringency subtraction will remove only perfectly matched sequences, whereas a low stringency subtraction will remove some tester strain sequences which have a relatively low base sequence homology with the driver DNA (Bjourson *et al.*, 1992). This is an important factor when considering the specific application of this technique.

1.11.4 Separation techniques and enrichment of target sequences

A variety of techniques to separate target DNA from tester-driver and driver complexes have been implemented. Many of the subtractive hybridisation methods developed thus far have been based on physical removal of the subtracted sequences (Straus & Ausubel, 1990; Bjourson *et al.*, 1992). These methods generally implement biotin-labelling of the driver DNA to allow physical removal via streptavidin-binding. The genomic subtraction technique employed by Straus and Ausubel (1990) implements physical separation and appears to be one of the more widely applied protocols (Seal *et al.*, 1992; Brown & Curtiss, 1996; Lan & Reeves, 1996; Schmidt *et al.*, 1998). A critical factor in physical separation protocols is that biotinylated DNA must bind reproducibly and quantitatively (Straus & Ausubel, 1990). The driver DNA must not be sterically hindered, which would render it unavailable for hybridisation (Bjourson *et al.*, 1992). Recently, the use of streptavadin-coated magnetic beads has facilitated removal of the subtracted sequences (Plum & Clark-Curtiss, 1994; Brown & Curtiss 1996; McGowan *et al.*, 1998; Schmidt *et al.*, 1998). Hydroxyapatite chromatography has also been used for physical separation (Lamar & Palmer, 1984; Wieland *et al.*, 1990). This technique separates double- and single-stranded nucleic acids but assumes that tester-specific sequences remain single-stranded and single-stranded driver sequences will not contaminate the single-stranded tester fraction.

Physical separation methods to enrich for target sequences either use a single round of competitive hybridisation (Lamar & Palmer, 1984) or multiple rounds of subtraction (Straus & Ausubel, 1990). Published enrichments using single-step competitive hybridisation have shown enrichments of less than 100-fold (Lamar & Palmer, 1984; Kunkel *et al.,* 1985, cited by Straus & Ausubel, 1990; Nussbaum *et al.,* 1987, cited by Straus & Ausubel, 1990). In Lamar and Palmers' (1984) method, an excess of sheared driver DNA is denatured with *Sau*3A-digested tester DNA and allowed to renature. Fragments that recreate *Sau*3A sticky ends are cloned into an appropriate vector. With methods such as this, the enrichment obtained can, in theory, be no greater than the ratio of tester to driver DNA (Straus & Ausubel, 1990; Wieland *et al.,* 1990). In contrast, Straus and Ausubel reported average enrichments of approximately 10-fold per round of subtraction. Therefore, by introducing multiple rounds, the level of enrichment can be increased significantly. More recent studies implement this strategy, as opposed to competitive hybridisation.

Methods have also been developed that rely on the principles of kinetic enrichment and PCR amplification (Lisitsyn *et al.*, 1993; Tinsley & Nassif, 1996; Akopyants *et al.,* 1998). Kinetic enrichment is based on the second-order kinetics of self-reassociation, originally proposed by Wieland *et al.* (1990) (cited by Lisitsyn *et al.,* 1993). In theory, if a population of DNA fragments containing a target subpopulation enriched n times relative to the unenriched fragments of the tester is melted and reannealed so that only a small proportion of double-stranded tester DNA forms, double-stranded target DNA would be present n^2 times relative to the other sequences present as duplex DNA (Lisitsyn *et al.,* 1993).

Methods using kinetic enrichment may have an advantage over those which employ physical separation to enrich for a double-stranded tester forms because physical separation is not completely reliable (Lisitsyn *et al.,* 1993). Bjourson *et al.* (1992) stressed the importance of using more than one physical separation technique to enrich for cell-specific nucleic acid sequences. These methods also seem to require less starting product than conventional physical separation and appear less unwieldy (Diatchenko *et al.,* 1999). Due to the combined use of kinetic enrichment and PCR amplification, the target sequences are sufficiently

enriched after only one round of subtraction. This streamlines the subtractive hybridisation technique considerably. Protocols that apply kinetic enrichment and PCR amplification as the means of enriching target sequences will be discussed more thoroughly in Section 1.12.

1.11.5 Adaptors and PCR

Many of the current subtractive hybridisation protocols implement the use of adaptors. These specifically designed oligonucleotides, ligated to the ends of tester DNA, facilitate PCR amplification. If multiple rounds of subtraction are used, the target DNA is present in very low concentrations, due to loss of material at each step of the procedure (Schmidt *et al.,* 1998). PCR is utilised to amplify the remaining DNA. In the Straus and Ausubel protocol, adaptors were ligated to DNA in the unbound fraction after multiple rounds of subtraction. Straus and Ausubel tried ligating the adaptors to the tester DNA before subtraction but found that this lowered the level of enrichment. Bjourson *et al.* (1992) ligated both *Sau*3A-digested tester and driver DNA to adaptors and introduced PCR before and after the subtraction process. By doing so, they suggested that a large molar excess of driver DNA could be provided and was easily renewable by PCR. Only a small amount of tester DNA is necessary for amplification and can be can be used directly as a probe, after the removal of subtracted sequences.

Adaptors used in protocols where kinetic enrichment is implemented are not only the means for PCR amplification, but the means of selectively enriching target sequences (Lisitsyn *et al.*, 1993; Tinsley & Nassif, 1996; Akopyants *et al.*, 1998). In kinetic enrichment protocols, adaptors are ligated only to tester DNA. Only self-annealed tester molecules have 5´-adaptors at each end of the duplex and can be filled in at both 3´ ends. Therefore, only self-annealed tester can be amplified by PCR at an exponential rate and is enriched relative to other tester DNA after amplification (Lisitsyn *et al.*, 1993).

1.11.6 Cloning the end-product

The target sequences isolated after subtraction are usually cloned by insertion into a suitable vector. This is either for screening of genomic libraries to identify and facilitate isolation and identification of particular nucleic acid fragments or to provide a renewable source of fragments for repeated use as probes (Bjourson *et al.*, 1992).

1.12 DNA-based subtractive hybridisation

As already mentioned, there are numerous variations in subtractive hybridisation protocols. Some specific protocols and the problems to which they have been applied are discussed below.

1.12.1 A physical separation protocol

Straus and Ausubel (1990) developed their technique of genomic subtraction to efficiently isolate DNA in a parent yeast strain, T1753, that corresponded to a 5 kb deletion in a laboratory-derived mutant. Briefly, the method involved isolating DNA from the deletion mutant (driver) and the parent (tester). An excess of sheared, biotinylated driver DNA was denatured in the presence of a small amount of digested tester DNA and allowed to reassociate. Tester DNAs that corresponded in chromosomal position to the deletion in the driver had no biotinylated complementary strand to bind. Both homogenous biotinylated DNA (both 5´ ends labelled) and heterogenous biotinylated DNA (one strand labelled) were removed by avidin-coated beads. Multiple subtraction rounds were required to ensure all complementary DNA was removed. Unbound DNA was ligated to adaptor-primers to allow PCR amplification of the remaining DNA. The DNA recovered at the end of the procedure corresponded to the DNA deletion in the mutant. No products larger than about 700 bp were amplified suggesting that small fragments in the complex mixture of template molecules were preferentially amplified. One of the important aspects of the Straus and Ausubel technique was that multiple subtraction rounds were required. For their experiment,

they showed three rounds of subtraction provided sufficient enrichment to accurately identify clones containing sequences that are absent in the deletion mutant. Clones that contained a fragment corresponding to the deletion were detected by hybridisation with a labelled probe. There was no mention of the likelihood of generating false positives.

1.12.2 Suppression subtractive hybridisation

Suppression subtractive hybridisation (SSH) was originally developed to study gene expression in eukaryotes (Diatchenko *et al.*, 1996). The central feature of SSH technique is the suppression PCR effect, which is mediated by long inverted terminal repeats (LITR). Siebert *et al.* (1995), reported that when the LITR are attached to the ends of DNA fragments, a stable panhandle-like structure is formed (Figure 1.1). In mRNA-based subtraction hybridisation, the suppression PCR effect was exploited to normalise cDNAs within a target population (Diatchenko *et al.*, 1996). More recently, the concept has been applied to DNA-based subtractive hybridisation (Akopyants *et al.*, 1998).

The panhandle-like structure inhibits exponential PCR amplification, due to being more stable than the primer-template hybrid. If PCR products are generated which contain double-stranded adaptor sequences at both ends, the ends of the individual DNA strands form a panhandle structure following every denaturation step (Siebert *et al.*, 1995). Exponential amplification can only occur when a hybrid is formed with a different adaptor at each end, thus suppressing what is being amplified. By incorporating the suppression effect in PCR, undesirable DNA fragments can be eliminated from a mixture of target sequences (Diatchenko *et al.*, 1999).

1.12.2.1 PCR-based subtractive hybridisation

The method of subtractive hybridisation implemented by Akopyants *et al.* (1998) utilises kinetic enrichment combined with PCR. DNA is first isolated from the respective strains and are *Sau*3A-digested. Driver DNA was supplied in excess and the two pools of DNA

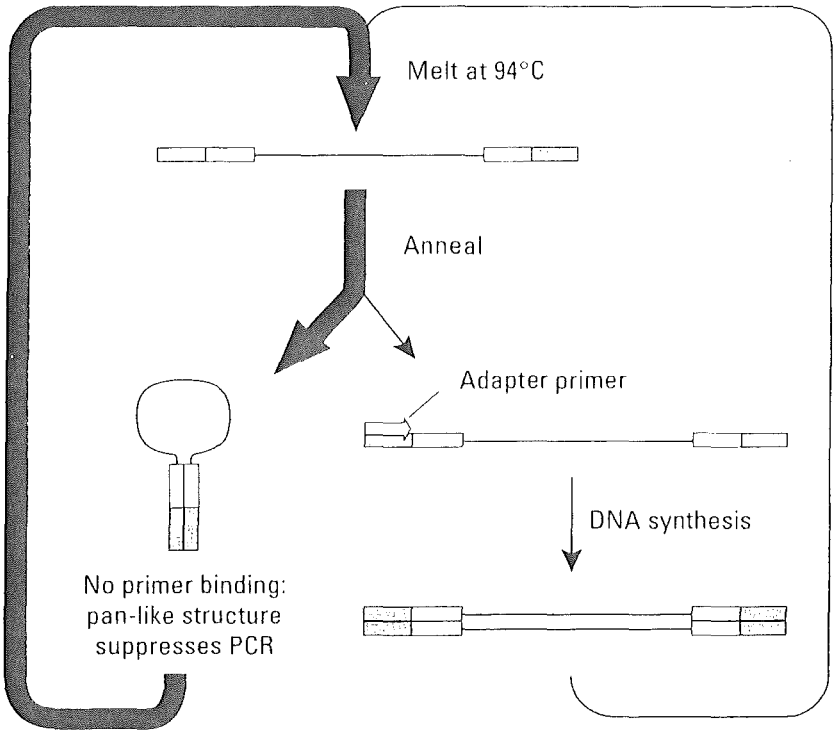


Figure 1.1 The PCR suppression effect (from Siebert *et al.*, 1995). PCR is suppressed when identical adaptors are present on each end of the target DNA.

were denatured and allowed to hybridise. PCR with specially designed adaptor-primers is utilised as the means of selecting for unique sequences.

Akopyants *et al.* (1998) tested their method of PCR-based subtractive hybridisation using isogenic *H. pylori* strains that differed only by the presence or absence of the 37 kb *cag* pathogenicity island (this is equivalent to about 2% of the *H. pylori* genome). Their results indicated that more than 90% of the cloned subtracted DNA were derived from the *cag* PAI. The technique was then tested on two unrelated *H. pylori* strains. Of the clones tested by hybridisation, 30 of 64 were judged to contain tester-specific sequences. Akopyants *et al.* suggested that other subtractive methods have been technically unwieldy and narrowly selective. Generally only a subset of strain-specific DNA molecules are obtained and DNA segments with potentially interesting mixes of strain-specific and common sequences are excluded (Akopyants *et al.*, 1998). Half of the clones obtained by Akopyants *et al.* contained patches of sequence that matched the driver, which they suggested may be important phenotypically.

In the PCR-based subtraction hybridisation used by Akopyants *et al.*, (1998) two different adaptors were ligated to two separate aliquots of restriction endonuclease digested tester. These aliquots were denatured and allowed to anneal with excess driver. In this step, the hybridisation kinetics led to equalisation and enrichment of target sequences among single-stranded tester molecules (Diatchenko *et al.*, 1999). Single-stranded tester DNA forms in the tester fraction were significantly enriched for target sequences as non-target DNA form heterohybrids with the driver. The separate aliquots were pooled and more denatured driver is added to further bind tester sequences present in the driver genome. The addition of excess driver at this stage increases the extent (C_0t value) of the hybridisation, thereby enriching the fraction of target sequences that were templates for PCR amplification (Diatchenko *et al.*, 1999). The process of PCR-based subtractive hybridisation is illustrated in Figure 1.2.

Two sequential PCRs were performed in the PCR -based subtractive hybridisation method to amplify unique tester sequences. The primers used in these PCR are illustrated

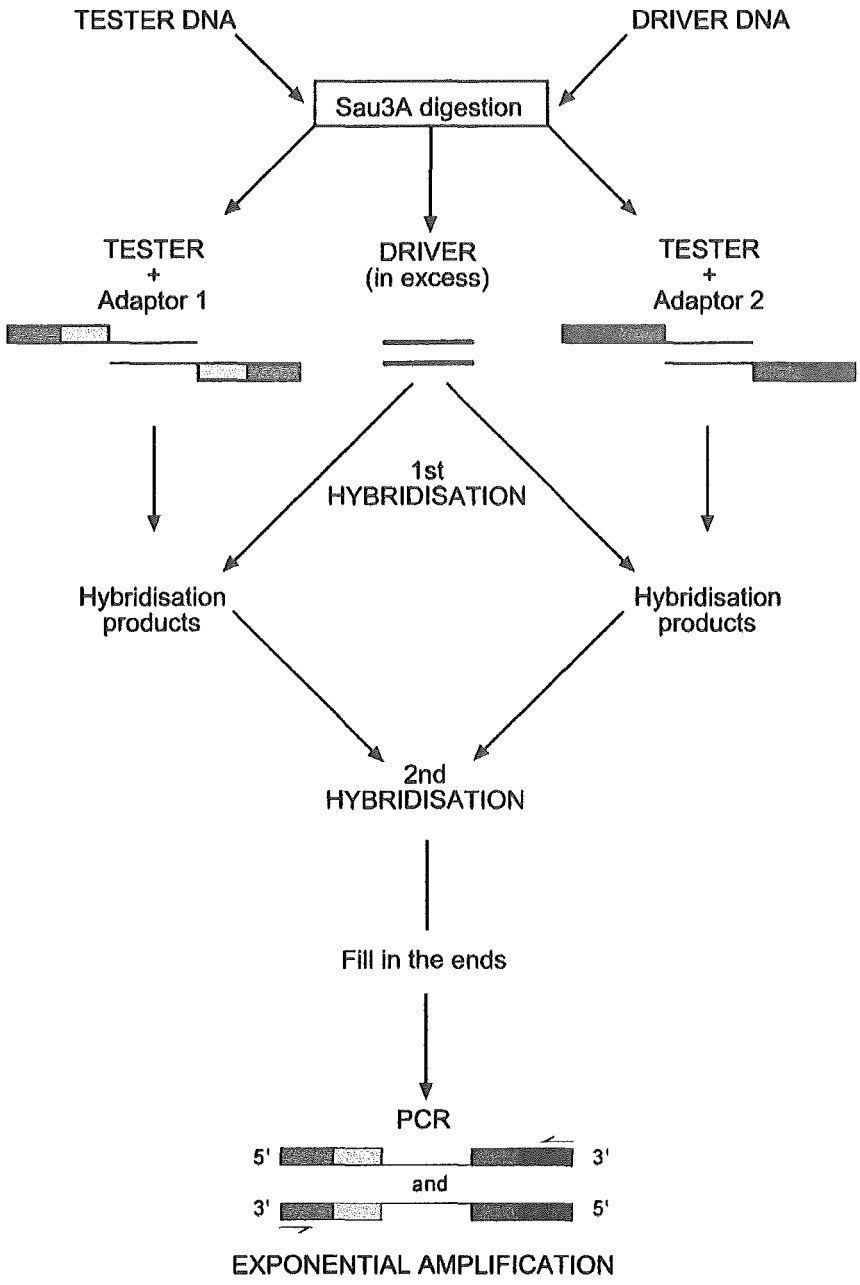


Figure 1.2 A schematic representation of the PCR-based subtractive hybridisation technique. The first hybridisation leads to enrichment of unique single-stranded tester molecules. The second hybridisation generates templates for PCR amplification of unique tester sequences. (Adapted from Akopyants *et al.*, 1998). See text for further detail.

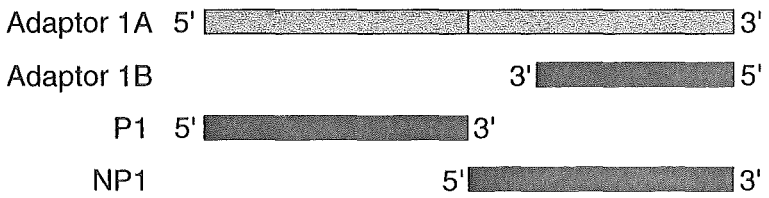


Figure 1.3 An illustration of the adaptor/primers used in the subtractive hybridisation protocol. The adaptor is ligated to the 5´ ends of *Sau*3A digested tester DNA. P1 matches the 5´ end of adaptors 1 and 2. NP1 matches only the internal portion of adaptor 1.

diagrammatically in Figure 1.3. The first PCR used the primer P1. This primer matched the long strand of adaptors 1 and 2 at their 5´ ends (Akopyants *et al.,* 1998). During the first round of PCR with the P1 primer, the adaptor ends were filled in using the klenow fragment, creating the complementary primer-binding sites required for amplification (Diatchenko *et al.,* 1999). The second PCR used primers NP1 and NP2. These nested primers matched the internal portions of the long strand adaptors 1 and 2, respectively (Akopyants *et al.,* 1998). The products from the nested PCR were inserted into an appropriate vector for cloning. Using a mathematical model, the enrichment for target sequences by the SSH procedure is estimated at over 1000-fold during one round of subtraction (Gurskaya *et al.,* 1996, cited by Diatchenko *et al.,* 1999). This estimation was supported in a model experiment with artificial targets (Diatchenko *et al.,* 1996).

1.12.3 Representational difference analysis

Representational difference analysis (RDA) is a variation of the subtractive hybridisation technique and was originally developed for eukaryote studies to determine the differences between two complex genomes (Lisitsyn *et al.,* 1993). In RDA the DNA complexity of the tester and the driver genomes was lowered by the preparation of a representative portion of each genome. The DNA was cleaved with rare-cutting restriction endonucleases and ligated to oligonucleotide adaptors for PCR amplification. After 20 rounds of PCR only fragments below one kilobase pair are amplified. Thus, a representative portion of the whole genome was obtained (Lisitsyn *et al.,* 1993). RDA applies kinetic enrichment as the means of isolating unique tester sequences.

Tinsley and Nassif (1996) adapted the method of Lisitsyn *et al.* to search for genes present in *N. meningiditis* but absent in *N. gonorrhoeae.* Due to the lower complexity of microbial genomes in comparison to eukaryote genomes, the initial PCR to gain representational amplicons was not performed. The subtraction process is similar to that of SSH, however, the suppression PCR effect was not exploited as a means of selecting for unique sequences and two rounds of subtraction were necessary (Tinsley & Nassif, 1996). 86% of clones

generated using RDA corresponded to distinct sequences in the meningococcus (Tinsley & Nassif, 1996). Perrin *et al.* (1999) also applied RDA to identify regions of the *N. meningiditis* and *N. gonorrhoeae* chromosomes which are specific to the pathogenic *Neisseria* species. It is interesting to note that a subtractive hybridisation technique has been applied in these cases to identify differences between species. Subtractive hybridisation is usually limited to intra-species comparisons as comprehensive analysis of chromosomal divergence is best when strains do not differ in more than about 10% of their genomic DNA content (Schmidt *et al.,* 1998). Inter-species comparison was possible in *Neisseria* spp. as estimates based on DNA-DNA hybridisation suggest degrees of homology of primary DNA sequence between 80 and 90% (Tinsley & Nassif, 1996).

1.13 Differential expression of genes

Methods comparing the differential expression of genes between one organism cultured using two sets of conditions, or two strains or species, have great potential for application in studies of virulence. A pathogen must be able to regulate particular genes required for survival in the different environments to which it may be exposed. A number of studies have demonstrated that expression of many bacterial genes is induced in response to environmental conditions, including genes for stress proteins and for virulence determinants (Bhagwat & Keister, 1992; Plum & Clark-Curtiss, 1994; Utt *et al.,* 1995; McGowan *et al.,* 1998).

The genes required for pathogenesis will most likely be different to those required for replication in the laboratory environment. Different strains or species may express different genes. This in turn may mean one strain is a more successful pathogen than another in different environments. It can be advantageous to study differences in gene expression, as opposed to genomic differences, when investigating virulence problems. These methods make it possible to identify avirulent variants that have arisen from spontaneous mutations in which a gene is present in the wild type and the mutant, but is inactive in the mutant (Utt *et al.*, 1995).

A number of methods have been developed to investigate differential gene expression. A simplistic method of differential hybridisation has been used to observe differential expression of genes between two strains of *Bradyrhizobium japonicum* under different conditions (Bhagwat & Keister, 1992). In this method RNA isolated from induced cultures was radiolabelled and used to directly probe restriction endonuclease digested genomic DNA from the respective strains. An excess of RNA from an uninduced culture was used to block the DNA during prehybridisation and hybridisation, preventing hybridisation of the radiolabeled probe (Bhagwat & Keister, 1992). Several clones were isolated showing strain-specific gene expression. This simple competitive hybridisation technique appears to have been superseded by more robust techniques for studying differential gene expression. These include subtractive hybridisation, differential display, and arbitrarily-primed PCR (AP-PCR). I will briefly summarise these methods and the problems to which they have been applied below.

1.13.1 RNA-based subtractive hybridisation

RNA-based subtractive hybridisation has been developed to identify genes that are expressed under defined conditions (Quinn *et al.*, 1997).

Plum and Clark-Curtiss (1994) were one of the first to utilise a RNA-based subtractive hybridisation method to demonstrate differential gene expression. They developed the method to identify *Mycobacterium avium* genes that are expressed by the bacilli living within macrophages to determine whether specific genes of *M. avium* were induced to express products that facilitate adaptation to conditions in the phagosome. Briefly, the method involved isolation of mRNA from two cultures. One culture was *M. avium* grown in broth (driver) and the other was a macrophage culture from which *M. avium* bacilli were harvested (tester). cDNA was then synthesised and adaptors ligated to allow PCR amplification. The subsequent steps follow the protocol of Straus and Ausubel (1990). One fragment was identified that coded for an mRNA that was highly specific for *M. avium* in phagosomes. By combining the techniques of cDNA synthesis and subtractive

hybridisation, transcripts from genes that were specifically induced when *M. avium* was growing in macrophages were enriched while the majority of house-keeping genes were eliminated. Plum and Clark-Curtiss suggest that the approach reduces the number of genes to be analysed and increases the possibility of identifying genes that may express virulence determinants.

Several researchers have subsequently used similar methods of mRNA-based subtractive hybridisation (Utt *et al.,* 1995; McGowan *et al.*, 1998). McGowan *et al.* (1998) used a modified version of Plum and Clark-Curtiss' method to investigate acid-induced expression of genes in *H. pylori.* This led to the discovery of an LPS-associated gene whose expression is induced after exposure to acid pH. Utt *et al.,* 1995, demonstrated the method could discriminate the expression of just one gene in *Listeria monocytogenes.*

RNA-based subtractive hybridisation holds many advantages over similar DNA-based methods. A major disadvantage of the DNA-based method has been that the DNA sequences between two closely related species or strains may be too similar and DNA base differences would be difficult to identify (Utt *et al.*, 1995). In comparison to AP-PCR, subtractive hybridisation does not require trial and error steps for locating the most appropriate primers for the identification of the expressed gene differences (Utt *et al.* 1995). Due to the relative instability of RNA it is possible that short-lived or low copy number messages could be lost. It is also possible that low copy RNAs may not be detected (Plum & Clark-Curtiss, 1994; Utt *et al.,* 1995). However, these are potential problems with all RNA-based methods, although there is much research to address these problems. Possible solutions are discussed below.

Directional random oligonucleotide primed (DROP) global amplification of cDNA has been applied to subtractive cDNA cloning as a solution to the problem of bias towards high copy number mRNAs, found in many mRNA-based methods (Hampson *et al.,* 1996). The technique does appear very sensitive, capable of detection of mRNAs with an abundance of 0.01% (Hampson *et al.*, 1996). A phagemid subtraction protocol has been applied for the enrichment of moderately induced sequences (Konietzko & Kuhl, 1998).

The protocol uses low ratio hybridisation of driver to target sequences of interest, and back-hybridisation of the subtracted sequences with induced sequences to reduce the accumulation of false positive clones (Konietzko & Kuhl, 1998). Both techniques were developed for eukaryote systems and therefore exploit the poly-T tail of the eukaryote transcript. Neither have reportedly been applied to prokaryote studies of differential gene expression. Suppression subtractive hybridisation, discussed in section 1.12.2, includes normalisation of cDNA populations (Diatchenko *et al.,* 1999). Normalisation occurs because the annealing process generating homohybrid and heterohybrid cDNAs is faster for more abundant molecules.

1.13.2 Differential display

Differential display PCR (DD-PCR) was first developed by Liang and Pardee (1992) as a means to identify and isolate genes that are differentially expressed in various cells or under altered conditions. DD-PCR is based on the use of random primers to generate representative fingerprints. Differential gene expression can be observed by comparing presence or absence of bands between fingerprints. Up-regulation or down-regulation of genes between two strains or conditions, can also be observed. However, this original method was designed to take advantage of polyadenylate tails present on most eukaryote mRNAs, to anchor the 3′ primers for reverse transcription. The 5′ primer in theory should be 6 to 7 bp, to allow it to anneal at a high frequency to the 5′ ends of cDNA strands. However, a 10-mer was found to give specific DNA amplification under optimised PCR conditions (Liang & Pardee, 1992). Specific DNA amplification is vital as the generated cDNA fingerprint must be reproducible in order to validate this method. Differential display also has a strong bias towards high copy number mRNAs (Bertioli *et al.,* 1992).

1.13.3 RNA arbitrarily primed PCR

The problem of low level adenylation of mRNA in prokaryotes can be circumvented by RNA arbitrarily primed (RAP) PCR-based methods (Wong & McClelland, 1994; Fislage *et al.*, 1997). In these methods the mRNA can be reverse-transcribed using either single arbitrary primers or random hexanucleotide mixtures and the subsequent PCR reaction contains one or two arbitrary primers (Fislage *et al.*, 1997). However, the primer design and the low number of cDNA bands generated pose some difficulties in scanning a complete genome for differentially expressed prokaryotic sequences (Fislage *et al.*, 1997).

Fislage *et al.* (1997) described a general method for the design of non-anchored primers for use in RAP-PCR. They statistically calculated the optimal primer length and sequence required to provide comparable results in prokaryotes, similar to Liang and Pardee's method for eukaryotes. The primer set was designed and tested for the strain *Escherichia coli* DH5α. The design of the 3´ primer was based on the knowledge that *E. coli* genes possess an AT-rich 3´-end. Fislage *et al.* suggest statistical analysis of coding sequences allows selection of short oligonucleotide sequences with above average occurrence in the prokaryotic genome. The findings of Fislage *et al.* (1997) have since been applied by Gill *et al.* (1999) to study *E. coli* global gene regulation in response to heat shock. Their comparison of heat shock clones to control clones provided the strongest evidence in support of the use of this technique as a differential display screening tool but this was only possible because of the abundance of data pertaining to the heat shock response.

Despite the potential difficulties in optimisation of RAP-PCR, the method has been successfully applied to study differential gene expression in various prokaryote systems. It has been applied to the analysis of the SOS response in *S. enterica* serovar Typhimurium (*S. typhimurium*) (Benson *et al.*, 2000) and has led to the identification of a stress-inducible gene of *S. typhimurium* (Wong & McClelland, 1994).

1.14 Objectives of this study

There is a general paucity of knowledge of virulence determinants of *C. jejuni*. The incidence of gastroenteritis caused by *C. jejuni* increases each year, causing significant morbidity and loss of productivity. *C. jejuni* is ubiquitous in the environment and it is necessary to determine the exact threat posed to humans by environmental strains. Some PFGE data suggest that not all strains are equally pathogenic to humans, demonstrated by an incomplete overlap of PFGE types of strains isolated from humans and strains isolated from other sources. This data also shows some strains may be isolated from a number of sources suggesting these strains may be more universal than others.

In *Salmonella*, all strains are known to be pathogenic to differing degrees and share important virulence traits. In *E. coli*, horizontal gene transfer has repeatedly allowed transition from a commensal relationship to a pathogenic one, and therefore *E. coli* pathovars produce disease by unrelated mechanisms (Bäumler, 1997). *H. pylori* strains are classed type I or type II on the basis of disease pathogenesis and the acquisition of the *cag* PAI (Censini *et al.*, 1996). The possible presence or absence of genes of one strain compared to another may play a role in the ultimate pathogenesis of the respective strains.

The interplay between a pathogen, its host environment and the greater environment are highly complex. Anything that may provide the pathogen with an advantage in either environment may be important in its ultimate pathogenesis. *C. jejuni* has been shown to be heterogenous and the natural competency of this organsim and high rates of recombination suggest a largely non-clonal population. Large differences may occur between strains and these differences may be important in strain pathogenic potential. This project aims to implement methods which directly compare gene expression between two strains of *C. jejuni* or total genomic differences between two strains. By characterising the intra-species variation between strains it may be possible to further our understanding of the processes of infection by this organism.

1.14.1 Aims of this study

The primary aim of this study was to determine if some strains of *C. jejuni* are more pathogenic to humans than other strains. In order to fulfil this aim a suitable technique would have to be applied that would enable the elucidation of genes that are present in one strain (a more pathogenic strain) and absent in another strain (an 'avirulent' strain). For this purpose a subtractive hybridisation procedure was selected. The specific aims of this study were as follows:

- Apply a subtractive hybridisation technique to determine if this procedure can be applied to *C. jejuni* to investigate intra-species variation.
- Characterise fragments obtained from the subtractive hybridisation by cloning and sequencing.
- Determine the relevance of the cloned fragments to the pathogenicity of the strain by directed mutagenesis and cell-binding and internalisation assays, as time permits.

Chapter 2

Materials and Methods

2.1 Bacterial strains

The bacterial strains used in this study are listed in Table 2.1

Table 2.1 Bacterial strains

Collection number	Isolate name/reference	Species
KLC4000	DH5α	*E. coli*
KLC4132	NZRM1958; [NCTC11168]	*C. jejuni*
KLC4315	Penner HS:19 Serovar	*C. jejuni*
KLC4356	F38011; M. Konkel, Washington State University	*C. jejuni*
KLC4394	F38011Δ*ciaB* B5 Km^r; M. Konkel, Washington State University	*C. jejuni*
KLC5000	F38011Δ*gmhD* Km^r	*C. jejuni*

2.2 Buffers and media

Solutions and media used in this study were prepared as described in Appendices I and II.

2.2.1 Antibiotics and supplements

When necessary, antibiotics and colorimetric reagents were added to liquid or solid media, in order to maintain selection pressure or identify plasmid constructs. Antibiotics and supplements used in this study are listed in Table 2.2.

Table 2.2 Antibiotics and supplements

Antibiotic/Supplement	Abbreviation	Final Concentration
Ampicillin	Amp	100 μg/ml
Cefoperazone	Cef	32 μg/ml
Deoxycholic acid	DCA	0.1% w/v
Tetracycline	Tet	30 μg/ml
5-bromo-4-chloro-3-indoyl -ß-D-galactopyranoside	X-gal	40 μg/ml
Isopropyl-ß-D-thiogalactoside	IPTG	100 μg/ml

2.2.2 Sterilisation of media

Heat sterilisation of all media, glassware, and other equipment by autoclaving was carried out at 121°C with 120 kPa of pressure for 20 min.

Reagents and antibiotics unable to tolerate autoclaving were filter sterilised using sterile 0.22 μm cellulose acetate membrane filters (Millipore Type GS).

2.3 Bacteriological methods

2.3.1 Culture conditions

C. jejuni isolates were incubated at 37°C in a microaerophilic environment (10% CO_2). Two types of media were used for culture maintenance. These were *Campylobacter* Blood-Free Selective Agar Base (Modified CCDA-Preston) (Oxoid CM739) with addition of cefoperazone (3.2 mg/l) and Mueller-Hinton Agar (MHA) (Remel) containing 5% defibrinated sheep's blood. MHA blood containing 0.1% DCA was used to induce gene expression in cultures, when required, for RNA extractions.

E. coli strains were incubated aerobically at 37°C. Cultures were grown in Luria Bertani broth (LB) or on LB plates containing 1.5% bacteriological agar (Oxoid).

2.3.2 Storage of strains

For short-term use, *C. jejuni* strains were maintained on CCDA containing Cef or MHA blood and passaged every 48 h to maintain culturability. For long-term storage, isolates were harvested from streak plates using brain heart infusion broth (BHI) (Merck) and glycerol in a 6:1 ratio. Three ml of BHI/glycerol was added to the plate and cells were dislodged with a sterile glass speader. The suspension was aliquoted into sterile 1.8 ml Nunc cryotubes and stored at –80°C until required.

E. coli strains were maintained on LBA plates and stored, short-term, at 4°C. For long-term storage cells were suspended in LB broth and glycerol (20%) and frozen at –80°C.

2.3.3 Sterilisation techniques

All manipulation of cultures was performed in an Email Class II Biological Saftey Cabinet. The cabinet was irradiated with microbiocidal UV light for 30 min and swabbed with diversol prior to use. General microbiological aseptic technique was applied to all experimental work.

Implements, such as glass spreaders, were surface sterilised with 70% ethanol and flamed. Metal loops were incinerated in a bunsen flame.

2.4 DNA isolation and manipulation

2.4.1 DNA isolation

The isolation of genomic DNA from *C. jejuni* was performed using the method of Pitcher *et al.* (1989). Briefly, cells were harvested from plates as already described (Section 2.3.2). Cells were pelleted by centrifugation at 3 700 × *g* at 4°C for 5 min. The pellet was resuspended in 0.5 of ml 0.85% NaCl, centrifuged at 14 800 × *g* for 1 min and resuspended in 0.1 ml TE buffer (pH 8.0). GES lysis solution (0.5 ml) was then added, and the mixture vortexed and incubated at 60°C for 15 min. 0.25 ml of ice cold 7.5M ammonium acetate

was added and the mixture incubated on ice for 10 min. This was followed by the addition of 0.5 ml chloroform and isoamyl alcohol (24:1 mixture). The extraction mixture tube was centrifuged for 13 min at 14 800 × *g* and the top phase removed to a separate Eppendorf tube. Isopropanol was added (0.54 V), mixed by inverting and incubated for 10–15 min at ambient temperature. The DNA was harvested by centrifugation at 14 800 × *g* for 3 min. The pellet of DNA was washed 5 times in 70% ethanol and vacuum dried. The pellet was resuspended in 0.1 ml ddH_2O with RNase A (20 µl/ml).

2.4.2 Estimation of genomic DNA quantity and purity

Quantity of genomic DNA was estimated by absorption spectroscopy. The spectrophotometer (Hitatchi U-2000) was calibrated using 1 ml distilled water. In a cuvette, 5 µl DNA was mixed with 995 µl ddH_2O. Absorbance was determined at 260 nm (A_{260}) and 280 nm (A_{280}). The A_{260} value was divided by 0.02 to give an estimation of the quantity (µg) of double-stranded DNA in 1 ml ddH_2O.

The purity of the DNA was estimated by the ratio of A_{260}/A_{280}. A ratio of 1.8 to 1.9 indicated highly purified DNA preparations (Sambrook, *et al.*, 1989).

2.4.3 Agarose gel electrophoresis of DNA

Tris-Acetate-EDTA (TAE) buffered gel electrophoresis was used to resolve PCR products and separate restriction endonuclease digested DNA fragments. Depending on the level of resolution required, 0.8–2% agarose gels were used. Agarose was dissolved by heating in 1× TAE buffer (Appendix II). Prior to heating, the gel weight was recorded and any weight lost during the dissolving process was made up by the addition of dH_2O.

DNA samples were mixed with 1–2 µl 6× DNA gel loading buffer (Appendix II) before loading into a pre-formed well. DNA fragments were separated by a current of 70–100 volts/cm^2. For a molecular weight reference, 3 µl of a 100 bp or 1 Kb plus molecular marker (Gibco BRL) were loaded alongside DNA samples.

Gels were stained with ethidium bromide (10 mg/ml in dH_2O) for 15–20 min with gentle agitation. Visualisation of gels was achieved using an Ultra Lum electronic UV transluminator (254 nm). Gels were photographed with a Kodak DC120 'Electrophoresis Documentation and Analysis' digital camera.

2.4.4 DNA elution from agarose gel

A QIAEX® II Gel Extraction Kit (Qaigen) was used to elute DNA fragments from agarose gels. The recommendations of the manufacturer were followed.

2.4.5 DNA ligations

DNA ligations for subtractive hybridisation experiments were performed with 1 µl T_4 DNA ligase (3 units/µl) (Promega) and 2 µl 10× DNA ligase buffer (Promega) in a final volume of 10 µl. All other DNA ligations used 1 µl DNA ligase with 5 µl 2× DNA ligase buffer (Promega) in a 10 µl reaction volume. Ligations using 10× DNA ligase buffer were incubated overnight at 12°C. Ligations using 2× DNA ligase buffer were incubated overnight at 4°C.

2.4.6 Plasmid precipitation

Ligated plasmids were ethanol precipitated using salmon sperm DNA as a carrier. A 10 µl reaction mixture containing 2 µl salmon sperm DNA (Gibco BRL), 5.5 µl 7.5M ammonium acetate and 2.5 µl dH_2O, was added to the ligation mixture (20 µl total reaction volume). This was followed by the addition of 3× volume ice cold 100% ethanol. The mixture was precipitated at –80°C for 20 min. The DNA was pelleted by centrifugation at 14 800 × *g* for 3 min at 4°C. The pellet was washed in 70% ethanol, air-dried and resuspended in 10 µl ddH_2O.

2.5 DNA-based subtractive hybridisation

The DNA-based subtractive hybridisation method was modified from the method of Akopyants *et al.* (1998). Little was altered from this original method except the amount of starting material was increased and later dilutions were excluded.

2.5.1 Endonuclease digestion of genomic DNA and ligation to adaptor-primers

10 μg genomic DNA was digested overnight with *Sau*3A and extracted by ethanol precipitation. The pellet was resuspended in 10 μl 10 mM Tris-HCl (1 μg/ml final concentration). Digested tester DNA (0.6 μl) (section 1.10) was aliquoted into two separate polymerase chain reaction (PCR) tubes. The two aliquots of tester DNA were ligated separately to the two adaptors, adaptor 1A/1B and adaptor 2A/2B (Table 2.4) (2 μM final concentration), in a final reaction volume of 10 μl. The ligation reaction was performed as in Section 2.5.5. To terminate the reaction 1 μl of 0.2M EDTA was added and the samples were heated to 70°C for 5 min.

2.5.2 Hybridisation

Three microlitres of driver DNA were added to 1 μl adaptor-ligated tester DNA, with 1 μl 5× hybridisation buffer (Appendix II). This was overlaid with mineral oil and denatured at 98°C for 1.5 min followed by hybridisation at 65°C for 1.5 h. The two samples were then combined and 1.5 μl of heat-denatured tester DNA was added with 1.5 μl 1× hybridisation buffer (Appendix II). This mixture was allowed to hybridise overnight at 65°C and stored at 4°C until use in subsequent PCR.

2.5.3 PCR using adaptor-primers

An initial PCR was performed using the primer P1 (10 μM) in a 25 μl total reaction volume. The reaction mix was incubated at 72°C for 2 min and then subjected to 25 cycles of 95°C, 30 sec; 66°C, 30 sec; 72°C, 1.5 min. The amplified products were then used in a nested PCR using primers NP1 and NP2 (Table 2.4) (10 μM each). The same PCR programme as above was used but the annealing temperature was increased to 68°C. The products from the nested PCR were ligated into the vector PGEM-T Easy for cloning and sequence determination.

2.6 Polymerase Chain Reaction

2.6.1 PCR reaction mix

All PCR were performed in thin-walled 1.5 ml Eppendorf tubes using a Corbett Research FTS-320 Thermal Sequencer. Oligonucleotide primers were purchased from Gibco BRL. Deoxynucleotides (dNTPs), buffers and *Taq* DNA polymerase were purchased from Roche. Prior to setting up the PCR, primers were diluted in sterile ddH_2O to 30 pmol/μl, and dNTPs were mixed in sterile ddH_2O to a 2mM concentration of each dNTP (dATP, dCTP, dGTP, dTTP).

Each PCR had a final volume of a 50 μl . The final concentrations for each reaction reagent are given. The reaction mix contained (unless otherwise stated): 3 pmol/μl each primer (forward and reverse), 0.4mM dNTPs (final concentration for each dNTP), 2mM $MgCl_2$, 10× DNA buffer and 2.5U *Taq* DNA polymerase. 2 μl of template DNA was added to the mix and the volume was made up to 50 μl with sterile ddH_2O. The mixture was briefly pulsed in a microfuge to collect all components of the reaction and overlaid with 3 drops of mineral oil.

2.6.2 PCR programmes

Various programmes were used in this study, depending on the target nucleic acid fragment to be amplified and the annealing temperature of the primers used.

A general programme consisted of 30 repeated cycles, each cycle consisting of a denaturation step (94°C for 1 min), a primer annealing step (45–55°C for 1 min) and an extension step (72°C for 3 min). Programmes concluded with a final soak step that held the amplicons at 4°C until storage at 4°C or –20°C.

2.6.3 Melting temperature (T*m*)

Primer annealing temperature is important in the PCR reaction as it determines amplicon specificity. Appropriate annealing temperatures were approximated in two ways. For nucleotides up to 18 bp, the T*m* was estimated by the following equation: (A+T)2° + (G+C)4°. Alternatively, the T*m* was estimated by subtracting 5–10°C from the T*m* (1M Na^+) value for the primer, provided by the manufacturer. The primer annealing temperature was optimised by experimentation.

2.6.4 PCR primers

A number of primers were used for different aspects of this study. The oligonucleotide primers used in this study are listed in Tables 2.3, 2.4 and 2.5.

2.6.5 Primer design - *flgE*

PCR primers amplifying a 350 bp fragment from *flgE* were designed on the basis of sequence alignments from five *flgE* sequences obtained from the EMBL database. The accession numbers for these sequences were AJ002074, AJ224790, AJ224791, AJ224792 and AJ224793. The programme ClustalX was used to align the sequences and to find conserved regions suitable for primer annealing. The putative *flgE* fragments were also aligned with

Table 2.3 General PCR primers

Code number	Primer name	Primer sequence	Reference
96-24	*lpxA*	5′-GCT CTA CTA AGT CTA TCT-3′	Ibbitt, 1997
96-25	*fabZ*	5′-GTG CGT CCT GGA GAT AGG C-3′	Ibbitt, 1997
97-01	*gmhA* (5′ internal)	5′-GAA TGG CAA GAA CAT CA-3′	Upritchard, 1997
97-02	*gmhA* (3′ internal)	5′-CTA GCC GTA TCA TCG CTT-3′	Upritchard, 1997
97-05	therm1	5′-TAT TCC AAT ACC AAC A-3′	Eyers *et al.*, 1993
97-06	therm2	5′-CGG TAC GGG CAA CAT TAG-3′	Eyers *et al.*, 1993
97-10	Sp6	5′-ATT TAG GTG ACA CTA TAG-3′	Promega
97-11	T7	5′-TAA TAC GAC TCA CTA TAG GG-3′	Promega
99-04	*ciaB* (458-478F)	5′-CAA ATT TAG ATG ATG CAA TGG-3′	Konkel *et al.*, 1999a
99-05	*ciaB* (974-995R)	5′-AAT TCA CAA TCT TCA AGT CC-3′	Konkel *et al.*, 1999a
99-06	*hupB* (13-32F)	5′-GAT TTC ATT TCA TTG TTG C-3′	Konkel *et al.*, 1999a
99-07	*hupB* (191-219R)	5′-TTT GAT TGT TTT TCC TGT GC-3′	Konkel *et al.*, 1999a
99-25	*gmhD* (319-338)	5′-ATA CGC GAA CAA TCA CCA-3′	Vasan, 1999
99-26	*gmhB/C* (1038-1019)	5′-ATA CGC GCA ACA TCA CCA-3′	Vasan, 1999
99-28	*gmhA* (2560-2587)	5′-GAG CTA TGC CTG CTA AAG CC-3′	Upritchard, 1999
00-59	*flgE*-F	5′-GCG GAT GGC TTT TTT ATG G-3′	This study
00-60	*flgE*-R	5′-CCC GTA TCG TTT TCA TCT T-3′	This study
	Multiplex	Due to the development of this set of primers for commercial application, details of these primers cannot be included	ESR/University of Canterbury

Table 2.4 Adaptors and primers for subtractive hybridisation (Akopyants *et al,* 1998)

Code number	Primer/adaptor name	Sequence
00-41	Adaptor 1A	5′-CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT-3′
00-42	Adaptor 1B	5′-AAC TGC CCG G-3′
00-43	Adaptor 2A	5′-CTA ATA GA CTC ACT ATA GGG CAG CGT GGT CGC GGC CGA GGT-3′
00-44	Adaptor 2B	5′-ACC TCG GCC G-3′
00-45	P1	5′-CTA ATA CGA CTC AC ATA GGG C-3′
00-46	NP1	5′-TCG AGC GGC CGC CCG GGC AGG T-3′
00-47	NP2	5′-AGC GTG GTC GCG GCC GAG CT-3′

Table 2.5 Primers for RAPD-PCR

Code number	Primer name	Primer sequence	Reference
96-13	ERIC-1R	5′-ATG TAA GCT CCT GGG GAT TCA C-3′	de Bruijn, 1992
96-14	ERIC-2	5′-AAG TAA GTG ACT GGG GTG AGC G-3′	de Bruijn, 1992
96-17	NR2	5′-CTG TAG TAA TCT TAA AAC ATT TTG-3′	Nachamkin *et al.*, 1993
97-24	REP2-1	5′-ICG ICT TAT CIC IGG CCT AC-3′	de Bruijn, 1992
97-25	REP1R-1	5′-III ICG ICG ICA TCI GGC-3′	de Bruijn, 1992

AJ224793. The programme ClustalX was used to align the sequences and to find conserved regions suitable for primer annealing. The putative *flgE* fragments were also aligned with AJ002074 to ensure the primer sites selected would encompass the region isolated by the subtractive hybridisation. The programme Oligo 6 was used to find suitable primers with an optimal annealing temperature and minimal likelihood of duplex and hairpin formation.

2.6.6 Controls

A negative control reaction was always included in each PCR assay. The control included all the PCR reagents but sterile ddH_2O was used to replace the DNA sample. Whenever necessary, a positive control containing a known DNA sample, was included.

2.6.7 RAPD-PCR

Randomly amplified polymorphic DNA (RAPD)-PCR was carried out under the following conditions. The PCR reaction mixture contained 0.3 µM each primer, 3 mM total $MgCl_2$, 5 µl 10× PCR buffer (with $MgCl_2$), 2µl DNA (in ddH_2O), 2.5 U *Taq* DNA polymerase and was made up to 50 µl total reaction volume with sterile ddH_2O.

The amplification programme used 36 cycles of 94°C for 1 min, 25°C for 1 min, 74°C for 4 min. A final cycle concluded with an elongation step of 74°C for 10 min to ensure completion of all initiated PCR products.

2.7 DNA sequencing

DNA sequencing of PCR products was achieved by the Sanger dideoxy sequencing method (Sanger *et al.,* 1999). Prior to sequencing, DNA samples were purified using a QIAquick™PCR Purification Kit (Quigen). DNA samples were sequenced at Waikato University using an ABI Prism 377 DNA sequencer and fluorescent dye terminator technology.

2.8 Southern hybridisation

2.8.1 Southern transfer

DNA of interest was electrophoresed through an 1% agarose gel and stained with ethidium bromide. An image of the DNA patterns was digitally captured beside a fluorescent ruler. DNA was transferred to a positively charged nylon membrane (Roche) using a Hoefer TransVac TE 80 vacuum blotter, attached to a Cole-Parmer Air Cadet vacuum/pressure station at ~15 kPa. Depurination, denaturation and neutralisation, were performed in accordance with the manufacturer's protocol. Transfer of single-stranded DNA was done using 20× SSC (Appendix II) transfer solution (90 min). Following the transfer, DNA was fixed to the membrane by crosslinking for 30 sec at 1200 kJ in an UVC-515 ultraviolet multilinker (Ultra Lum). To determine the efficiency of the transfer, the gel was restained with ethidium bromide and visualised on a UV transilluminator. The membrane was either probed immediately or blotted dry and wrapped in foil and stored at ambient temperature.

2.8.2 Probe labelling

Digoxygenin-dUTP (DIG) was used to non-radioactively label PCR products for use in Southern hybridisations. The PCR DIG Probe Synthesis Kit (Roche) was used, following the recommendations of the manufacturer, with some adjustments. The DIG-dUTP was diluted 1:1 with the stock dNTPs provided.

Agarose gel electrophoresis was used to confirm that the probe was successfully labelled by observing a shift in the relative migration distance of the labelled versus the unlabelled product. DIG-labelling of a PCR product inhibits its migration through the gel.

2.8.3 Hybridisation and label detection

The DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) was used for hybridisation and detection. Hybridisation was performed according to the procedure

outlined in the instruction manual. Briefly, a Hybaid tube containing 10 ml of DIG Easy Hyb (Roche) was prewarmed to 42°C. Pre-hybridisation was carried out for 30–60 min at 42°C with constant rotation in a Hybaid LTD Micro-4 HB-MCR4 oven. The probe was denatured for 10 min at 100°C and added to another 10 ml of prewarmed DIG Easy Hyb. The prehybridisation solution was replaced with the probe containing mixture and hybridisation was carried out overnight at 42°C with constant rotation.

Following hybridisation, the membrane was washed twice at ambient temperature for 15 min in 2× SSC, 0.1% SDS. The membrane was washed twice at 68°C for 15 min in 0.5× SSC, 1% SDS with constant rotation. After the washes the membrane was rinsed for 5 min in washing buffer and incubated in 100 ml blocking solution for 30 min at ambient temperature. Anti-DIG-AP conjugate (antibody) was diluted 1:10 000 in 1× blocking solution. The membrane was incubated for 30 min in 20 ml diluted antibody solution, washed twice for 15 min in washing buffer and equilibrated for 5 min in detection buffer. The membrane was placed on a sheet of acetate, DNA side up, and 20 drops of CSPD, ready-to-use solution was applied to the membrane. The membrane was immediately covered with another sheet of acetate and the CSPD was spread evenly over the membrane. After 15 min incubation at ambient temperature, excess liquid was squeezed out and the membrane was incubated for 15 min at 37°C to enhance luminescence. Probe hybridisation was visualised by exposing the membrane to Hyperfilm™ECL™ (Amersham Pharmacia Biotech) for 5 min at ambient temperature.

2.9 Cloning techniques

2.9.1 Preparation of competent cells

The *E. coli* strain DH5α was used for cloning experiments. Electrocompetent cells were prepared using a variation of the protocol supplied with the Bio-Rad Gene Pulser™ (Bio-Rad, Richmond, CA). Briefly, 3 ml of LB broth was inoculated from a single colony on an LB streak plate. The broth was incubated at 37°C overnight, before being added to 250 ml

of sterile LB broth. To obtain early to mid-log phase growth, cultures were incubated for 3 h at 37°C followed by 1 h in a 37°C shaking (200 rpm) water bath. Cultures were placed on ice for 20 min before being transferred to a sterile 250 ml centrifuge tube. All centrifugation was at 5 800 x *g* for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 100 ml ice cold ddH_2O. The pellet was resuspended in one-half the original volume of ice cold, sterile ddH_2O, centrifuged, and resuspened in 1/50 volume ice cold, sterile 10% glycerol. The cell suspension was transferred to an 1.5 ml Eppendorf tube, centrifuged and resuspended in 200 µl 10% glycerol. 50 µl aliquots of the final cell suspension were placed into Eppendorf tubes and stored at –80°C until required.

2.9.2 The cloning vector pGEMT®-Easy

The vector pGEMT®-Easy (Promega) was selected as the cloning vector in this study. It was selected because it was a convenient and reliable system for cloning PCR products. The vector contains T7 and Sp6 RNA polymerase promoters flanking a muliple cloning region within the α-peptide region of ß-galactosidase. Recombinant clones can be identified by colour screening on indicator plates, due to transcriptional inactivation of the α-peptide (Promega Technical Manual). The recommendations of the manufacturer were followed when using this vector.

2.9.3 Cleaning of electroporation cuvettes

The electroporation cuvettes (0.2 cm gap length) were cleaned just prior to electroporation. The cuvettes were first soaked for 10 min in concentrated bleach and rinsed in dH_2O. They were then soaked for 10–20 min in absolute ethanol followed by 10 min in 70% ethanol. After air-drying the cuvettes were thoroughly washed with 500 µl of sterile 10% glycerol and placed on ice until use.

2.9.4 Electroporation

Electroporation was carried out using a Gene Pulser™ (Bio-Rad). The settings were 25 μF, 2.5 kV and 250 Ω.

50 μl of frozen electrocompetent cells were thawed on ice and mixed with 2 μl of plasmid DNA and allowed to sit on ice for ~1 min. The mixture was transferred to an ice cold cuvette and pulsed at the settings above. Typically the time constant was 4.0–4.6 msec. 1 ml of SOC (Appendix II) was added to the cuvette immediately after the pulse and mixed thoroughly. The transformed cells in SOC were transferred to a sterile Eppendorf and incubated aerobically for 1 h at 37°C. Transformants were pelleted by centrifugation for 1 min at 1 000 × *g* and resuspended in 100 μl sterile ddH_2O. The cell suspension was spread plated onto LBA plates containing Amp, IPTG and X-Gal and incubated overnight at 37°C. White colonies were streaked onto fresh LBA plates containing Amp.

2.9.5 Transformation controls

To ensure ligation of the insert into the vector was successful, ligation efficiency was assessed using a plasmid with a high transformation efficiency. The plasmid was digested with *Hind*III and religated before transformation of DH5α. The transformation efficiency of the digested/ligated plasmid was compared to that of an undigested plasmid. A background control was included with all transformations. This control was the vector with no insert.

2.9.6 Plasmid extraction

A lysis by boiling method was used for plasmid extraction (Sambrook *et al.,* 1989). Cells were harvested by spreading 3 ml LB broth onto the plate and removing 1.5 ml to a sterile Eppendorf tube. The suspension was centrifuged at 12 000 × *g* for 2 min and the supernatant was removed by aspiration. The pellet was resuspended in 350 μl STET buffer (Appendix

II) and the tube was placed in boiling water for 1 min. Following centrifugation at 12 000 × *g* for 10 min at ambient temperature, the supernatant was removed to a clean and sterile Eppendorf tube. 40 µl 2.5M sodium acetate (pH 5.2) and 420 µl isopropanol were added to the supernatant, mixed by vortexing and incubated for 5 min at ambient temperature. Pellet recovery was by centrifugation at 12 000 × *g* for 10 min at 4°C. The pellet was air-dried and washed twice with 70% ethanol and air-dried. The pellet was resuspended in 50 µl sterile ddH_2O containing RNaseA (20 µg/ml). This method of plasmid isolation was sufficient for screening transformants by PCR.

2.10 RNA handling and manipulation

2.10.1 RNA extraction

Total RNA was extracted from all isolates using the RNeasy Plant Mini Kit (Qiagen). This method was chosen for its efficiency and reproducible results.

Cells were harvested by scraping plates with 3 ml ddH_2O. A 1 ml aliquot (approximately 10^8 CFU) was taken from each plate and centrifuged at 3 700 × *g* for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 100 µl of lysozyme-containing buffer (final concentration of lysozyme was 400 µg/ml) and incubated for 10 min at ambient temperature. Subsequent steps followed the protocol provided with Qiagen kit, using the buffers, spin columns and collection tubes provided.

Due to the potential for DNA contamination of the extracted RNA, samples were subjected to DNase I digestion prior to RT-PCR and subsequent PCR amplification. As a control some RNA from each extraction was digested with RNase A and both DNase I and RNaseA prior to RT-PCR.

2.10.2 RNA handling

In order to avoid introducing RNases into the RNA sample, the following precautions were taken. All glassware was treated with 0.1% dimethyl pyrocarbonate (DMPC) in water. This involved filling the glassware with 0.1% DMPC, allowing it to stand overnight at 37°C and autoclaving to remove traces of DMPC. Solutions were similarly treated. Where possible certified RNase-free disposable plasticware was used. Aseptic technique was applied when handling RNA. Powder-free latex gloves were worn at all times and changed frequently. Aerosol barrier tips were used for pipetting. RNA work was performed in a perspex cabinet specifically designed for RNA work. The cabinet was irradiated with UV for 30 min prior to use.

2.10.3 DNase and RNase digestions

It was important that RNA samples for subsequent RT-PCR were not contaminated with DNA or false positive amplification of the fragments of interest might have resulted. Therefore, total RNA from each extraction was divided into 10 µl aliquots. Three aliquots were subjected to digestion with DNase I, one aliquot was digested with RNase A and one aliquot was digested with DNase I and RNase.

The DNase digestion mixture consisted of 10 µl total RNA in DMPC-H_2O, 4.4 µl DMPC-H_2O, 2 µl 10× PCR buffer (Roche), 1.6 µl $MgCl_2$ (25 mM stock concentration) and 1 µl DNase I (30 units/µg). This mixture was incubated at ambient temperature for 15 min and the reaction was terminated by the addition of 0.5 µl 50mM EDTA. 500 µl isopropanol was added and the mixture was frozen at –80°C for at least 1 h. The mixture was centrifuged at 17 400 × *g* at 4° for 10 min and the subsequent pellet was washed with 70% ethanol and air dried. The pellet was resuspended in 10 µl DMPC-H_2O.

2.10.4 RNA quantification and purity

The quantity of RNA and the purity of the preparation was estimated by measuring the absorbance (with the UV lamp on) at A_{260} and A_{280}. To ensure cuvettes were free of RNase activity, they were washed with 0.1 M NaOH, 1 mM EDTA and DMPC-H_2O. DMPC-H_2O was used as a blank control. 10 µl of the DNaseI-treated RNA preparation was mixed with 990 µl DMPC-H_2O. An A_{260} value of 1 is approximately equal to 40 µg/ml of RNA. Approximately 10 mg of total RNA was isolated in each extraction.

An estimation of the purity of the RNA preparation could be gained by the ratio of A_{260}/ A_{280} in 10mM Tris.Cl (pH7.5). A ratio of 1.8 to 2.1 in 10 mM Tris.Cl is indicative of pure RNA (Qiagen RNeasy Mini Handbook).

2.10.5 RT-PCR

To be sure that mRNA was present, a two step Reverse Transcription-PCR (RT-PCR) was implemented. First strand cDNA synthesis was achieved using Superscript™II RNase H$^-$ Reverse Transcriptase (Gibco BRL) and random primers (Gibco BRL) with the 5× First-Strand Buffer and 0.1M DTT provided. The recommendations of the manufacturer were followed. Briefly, 5 µl DNase-digested RNA in DMPC-H_2O, 5 µl DMPC-H_2O and 1 µl random primers (30 ng/ml) were gently mixed in a PCR tube and incubated at 70°C for 10 min, followed by cooling on ice. 4 µl 5× First-Strand Buffer, 2 µl 0.1M DTT and 1 µl 10mM dNTPs were added to the tube and the mixture was incubated at 25°C for 10 min, followed by 42°C for 2 min. Finally, Superscript™II was added and the reaction was continued at 42°C for 50 min. The reaction was terminated by heating to 70°C for 15 min. Randomly primed cDNA was stored at –80°C until required for subsequent PCR amplification. In the second step, 10% of the first strand reaction was used for PCR.

An RT control was included with all RT-PCR. This control contained DNase-digested RNA and all other reagents but no Superscript™II was added to the reaction.

2.11 Real-time TaqMan® PCR analysis

TaqMan® PCR analysis was performed on cDNA samples to give an indication of sample integrity and quantity. Forward and reverse primers for 23S rDNA, *ciaB* and *lpxA* were used with the appropriate probes. The real-time TaqMan® PCR was performed on a ABI 7700 Sequence Detecter in conjunction with the programme Sequence Detecter Version 1.7.

Each reaction mixture contained 2 µl of cDNA sample and had a final volume of 25µl. Primers and probes to detect 23S rDNA, *ciaB* and *lpxA* were supplied by ESR, but the sequences cannot be divulged due to commercial sensitivity. The final concentrations of primers (forward and reverse) for amplification of 23S rDNA and *ciaB* were 900 nM each. The forward primer for *lpxA* was at a final concentration of 900 nM while reverse primer was 300 nM. The final concentrations of probes for 23S rDNA, *ciaB*, and *lpxA* were 225 nM, 125 nM and 175 nM, respectively. 12.5 µl TaqMan® Universal Master Mix (PE Applied Biosystems) was used in the reaction and the reaction volume was made up to 25 µl with sterile ddH_2O.

Chapter 3

Results

3.1 DNA-based subtractive hybridisation

In order to determine whether *C. jejuni* was amenable to analysis by subtractive hybridisation methodology, the PCR-based subtractive hybridisation method of Akopyants *et al.* (1998) was selected. Various DNA-based subtractive hybridisation methods have been applied to a wide range of bacterial species to detect genomic differences between strains (Brown & Curtiss,1996; Lan & Reeves, 1996; Tinsley & Nassif, 1996; Akopyants *et al.*, 1998; Schmidt *et al.*, 1998). The method applied by Akopyants *et al.* (1998) was chosen for its ease of utility, its relative low cost, and its prior application to *H. pylori*, an organism related to *C. jejuni*.

3.1.1 Control subtractive hybridisation

In order to determine the potential of DNA-based subtractive hybridisation to detect a known difference between strains, a control experiment was performed. For this experiment, two isolates were used that were isogenic, i.e., F38011 and F38011Δ*ciaB*. The isogenic mutant F38011Δ*ciaB* was constructed by disruption of the *ciaB* gene by homologous recombination using a truncated *ciaB* gene located on a colE1 type plasmid (pBIISK(+)), containing a *Campylobacter* kanamycin resistance gene. This plasmid is unable to replicate in *C. jejuni* and is therefore a suicide vector (Konkel *et al.*, 1999a). Consequently, the only difference between these strains is the presence of the kanamycin resistance gene, as well as the plasmid pBIISK(+). The products from a DNA-based subtractive hybridisation using

F38011Δ*ciaB* as the tester and F38011 as the driver (Figure 3.1), should contain fragments that correspond to the plasmid construct. To ascertain this, Southern hybridisation was performed.

The products from the subtractive hybridisation were transferred onto a nylon membrane. The probe for the Southern hybridisation was constructed from a plasmid-expressed copy of *ciaB*, using the primers T7 and T3 to amplify the *ciaB* construct from pBIISK(+) and labelled with DIG-dUTP. This construct should also contain the kanamycin resistance gene. The probe was approximately 2.3 kb. If the product from the subtractive hybridisation between F38011Δ*ciaB* and F38011 contained fragments of the kanamycin resistance gene as expected, the probe was predicted to hybridise to the 50 to 350 bp fragments observed in lane two of Figure 3.1. Unlike what was predicted, no hybridisation was observed. However, hybridisation to the the 1 kb plus DNA ladder was observed (data not shown). This experiment was repeated and the same results were obtained.

In this experiment, it would have been preferable to probe directly for the kanamycin resistance gene. Primers to amplify the kanamycin resistance gene were obtained. However, problems were encountered obtaining positive amplification with these primers (data not shown).

To ensure F38011Δ*ciaB* had not lost the kanamycin insert from the *ciaB* gene prior to the subtractive hybridisation, a Southern hybridisation was carried out on *Hin*dIII digested F38011 and F38011Δ*ciaB* genomic DNA. The respective digests were hybridised with a DIG-labelled amplicon of *ciaB*, amplifed from F38011 DNA with primers 99-04 and 99-05 (Table 2.3). An unlabelled amplicon generated with these primers is approximately 400 bp (results not shown). The probe hybridised to a 3 kb fragment and a 1 kb fragment in both F38011 and F38011Δ*ciaB* (Figure 3.2). In F38011Δ*ciaB*, the probe hybridised a fragment of approximately 1.8 kb in size that was not hybridised in F38011. The sizes of the hybridised fragments are as expected from the restriction maps of F38011 and F38011Δ*ciaB,* respectively (Konkel *et al.,* 1999a). The differential hybridisation of the 1.8 kb fragment in F38011Δ*ciaB* confirms that the kanamycin resistance gene is maintained

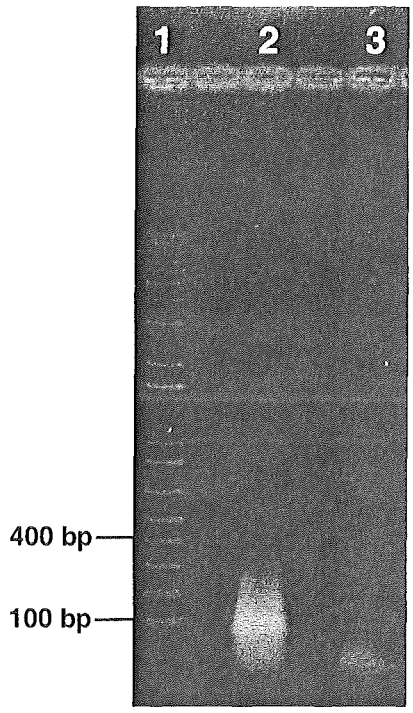
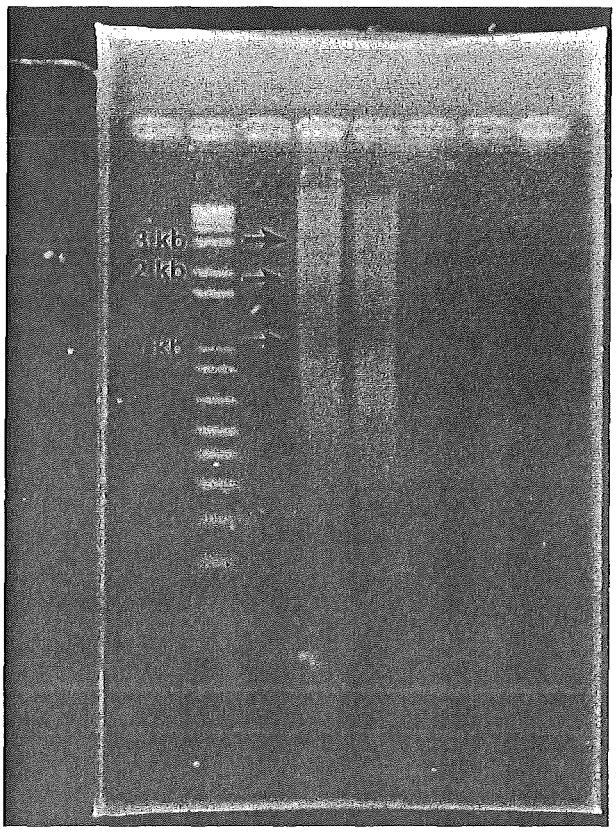


Figure 3.1 Fragments resulting from a subtractive hybridisation using F38011Δ*ciaB* as the tester and F38011 as the driver. Lane 1, 1 kb plus DNA ladder; lane 2, fragments from subtractive hybridisation; lane 3, negative control.

A



B

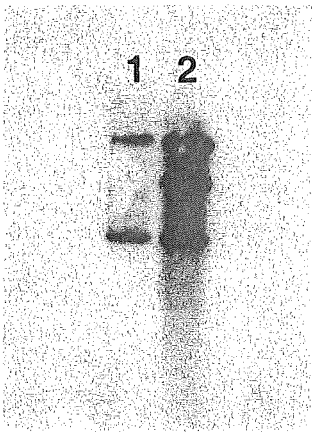


Figure 3.2 Southern hybridisation experiment to confirm the presence of the kanamycin resistance gene in F38011Δ*ciaB*. Part A shows *Hin*dIII digested genomic from F38011 (lane 1) and F38011Δ*ciaB* (lane 2) beside the 1 kb plus DNA ladder (M). The arrows indicate the positions of the hybridised fragments from the Southern hybridisation, shown in Part B.

in F38011Δ*ciaB* and therefore should have been able to be detected using subtractive hybridisation.

That the kanamycin resistance gene was not detected in the product from the subtractive hybridisation suggests that either the subtractive hybridisation method has not selectively enriched for sequences present in F38011Δ*ciaB* or that detection was not possible with the probe that was used. The probe used to detect the presence of the kanamycin resistance gene amongst the subtractive hybridisation product was large (2.3 kb), while the fragments to be detected may have been small. It may be possible that the kanamycin resistance gene was indeed present in the subtractive hybridisation product, but the fragments present were too small to efficiently hybridise the large probe. There may also have been considerable background DNA fragments in the product which may have inhibited probe-binding.

Due to the inconclusiveness of the control subtractive hybridisation and to further test the application of the DNA-based subtractive hybridisation, an experiment was carried out between two different strains, NCTC11168 and F38011.

3.1.2 Subtractive hybridisation between F38011 and NCTC11168

To test whether the DNA-based subtractive hybridisation technique may be applied to find differences between two different strains of *C. jejuni,* the method was tested on the strains F38011 and NCTC11168, both human gastroenteritis isolates. These strains were selected for this experiment as they are both well characterised. F38011 has been used extensively for research in this laboratory and other laboratories (Konkel *et al.,* 1998; Konkel *et al.,* 1999a; Konkel *et al.,* 1999b), whereas the full genome sequence is available for NCTC11168 (Parkhill *et al.*, 2000), providing an ideal reference source for any 'unique' sequences that might be isolated using this technique. Macrorestriction profiling using PFGE of the two strains generated unique profiles, although conservation of some gene sequences, eg., the *lpxA* gene has been observed on the same sized fragment in the respective

profiles (Knibb, 2001). Additionally, eight allelic differences in *lpxA* from NCTC11168 compared with F38011 were observed (Knibb, 2001). F38011 is also known to possess the *lex2B* gene which is absent from NCTC11168 (Jankovic, 1999).

For the purpose of this subtractive hybridisation experiment, NCTC11168 was selected for use as the driver strain and F38011 was used as the tester strain. Therefore, the DNA fragments isolated should relate to sequences that are present in NCTC11168 but are absent in F38011. This in turn would enable direct sequence comparison of the isolated fragments to the NCTC11168 seqence database.

An initial subtractive hybridisation was performed using 12 ng of tester DNA and 600 ng of driver DNA (a 1:50 ratio) (Akopyants *et al.,* 1998). However, the first trial of this technique yielded no visible end products on a 2% agarose gel (results not shown). It was reasoned that DNA may have been lost during ethanol precipation and therefore was not detected in subsequent reactions. For this reason the starting DNA concentrations were increased 10-fold and two subtractive hybridisations were performed in tandem. Aliquots from one of these subtractive hybridisations were taken at each step and tested for the presence of DNA by agarose gel electrophoresis. When the starting DNA concentration was increased, DNA was observed at each step of the subtractive hybridisation (results not shown). In retrospect, altering the tester to driver ratio and/or other conditions of the subtractive hybridisation, may have been preferable to increasing the starting DNA concentration. This will be discussed more fully in Section 4.3.

The products from the subtractive hybridisation using NCTC11168 as the driver and F38011 as the tester were cloned in pGEMT-Easy. After cloning, the unknown DNA fragments were amplified from the vector with the primers T7 and Sp6 (Figure 3.3). Five of these fragments were sequenced for further analysis. These fragments were labelled SH1-1 to SH1-5.

To ensure the reproducibility of the subtractive hybridisation procedure, a second subtractive hybridisation was performed in the same way as this first subtractive hybridisation that

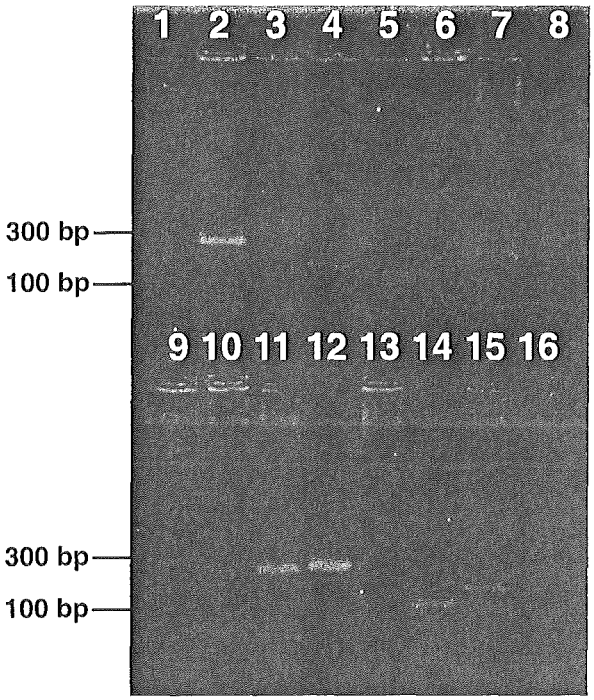


Figure 3.3 Cloned fragments from a subtractive hybridisation between NCTC11168 and F38011 amplified from the pGEMT-Easy vector using primers T7 and Sp6. Lanes 1 and 9 contain the 1 kb plus DNA ladder. Lane 8 contains the product amplified from the vector with no insert. Lane 16 is the negative control. The products resolved in lanes 2, 7, 10, 11 and 12 were analysed further.

yielded visable end products. Again, an increased starting concentration of DNA was used. Six fragments from the second subtractive hybridisation were sequenced. These were labelled SH2-1 to SH2-6.

3.1.3 Sequence analysis

Sequence analysis revealed that all fragments except SH2-3 were flanked by *Sau*3A restriction sites adjacent to adaptor/primer sequences (Figure 3.4). SH2-3 was flanked by adaptor/primer sequences but no *Sau*3A restriction sites. This may be explained by partial DNase digestion taking place in the reaction. The sequences between the *Sau*3A sites were very short, only 49 to 67 bp in length. These results may be explained by preferential amplification of short sequences. This will be discussed more fully in Section 4.3.

Searches of *Campylobacter* sequences located in Genbank were performed on all sequenced fragments. Blastn searches revealed all sequenced fragments had 100% nucleotide identity with the genome sequence for NCTC11168 (Table 3.1). This Blastn result suggests that the isolated fragments originated from the tester strain, NCTC11168. Blastx searches for the five sequenced fragments from the first subtractive hybridisation revealed SH1-1 and SH1-5 shared significant identity at the amino acid level with a *C. jejuni* NCTC11168 A/G-specific adenine glycosylase, with 100% identity. SH1-2, SH1-3 and SH1-4 shared significant identity with the *C. jejuni* FlgE protein, a component of the flagellar hook chain protein. All three sequences were identical at the amino acid level (Table 3.1). This result was deemed reasonable, due to FlgE being encoded by a hypervariable region of the *C. jejuni* genome (Lüneberg *et al.*, 1998; Parkhill *et al.*, 2000).

Four fragments from the second subtractive hybridisation, SH2-1, SH2-2, SH2-4 and SH2-5, also produced statistically significant alignments at the amino acid level with FlgE (100% identity) (Table 3.1). Sequence alignments using the progamme ClustalX revealed fragments SH1-2, SH1-4, SH2-2, SH2-4 and SH2-5 were identical. SH1-3 and SH2-1 were different from the other *flgE* sequences but were identical to each other. However,

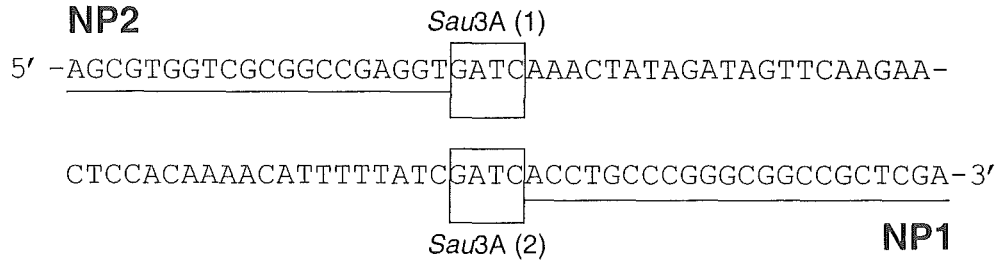


Figure 3.4 An example of a *flgE* fragment flanked by sequences corresponding to the primers NP1 and NP2 (underlined and labelled respectively).These primers were used for the final nested PCR in the subtractive hybridisation. The *Sau*3A restriction sites are also indicated by a box.

Table 3.1 Summary of Blast results.

Fragment name	Blastn identity to NCTC11168	Blastx identity to NCTC11168	Gene represented	Putative/known function
SH1-2 SH1-3 SH1-4 SH2-1 SH2-2 SH2-4 SH2-5	100%	100%	*flgE*	Flagellar hook protein
SH1-1 SH1-5 SH2-6	100%	100%	*mutY*	A/G-specific adenine glycosylase
SH2-3	100%	100%	*sdaA*	L-serine dehydratase

when the sequence represented by SH1-3 was reversed and complemented, and subsequently aligned, the sequence matched those of the other *flgE* sequences (Figure 3.6). This would have been due to the orientation of the products within the cloning vector. Similarly, when sequence for SH2-6 with 100% identity to an A/G-specific adenine glycosylase (Table 3.1), was reversed and complemented, it completely aligned with SH1-1 and SH1-5.

A third sequence, represented by SH2-3 showed 100% identity to a *C. jejuni* NCTC11168 L-serine dehydratase (Table 3.1).

3.1.4 *flgE*

Due to the high frequency of isolated fragments giving significant alignments with FlgE, further investigation of *flgE* was warranted. Primers were designed to amplify a region of the *flgE* gene from NCTC11168 and F38011. These primers generated an amplicon of approximately 350 bp, as expected (Figure 3.5). The sequences from the respective amplicons were aligned with *flgE* fragments isolated from the subtractive hybridisations. The alignment with the *flgE* amplicon from F38011 showed one base substitution between the *Sau*3A restriction sites (Figure 3.6). The data indicates that the subtractive hybridisation technique implemented here is highly reproducible, as the same base substitution was isolated several times and in two independent experiments. This is supported by isolation of more than one identical fragment with similarity to an adenine glycosylase. The ability of the technique to detect a single base substitution may also suggest the subtractive hybridisation technique is highly discriminatory. However, if this were the case one might expect many other different fragments to have been isolated. For example *lpxA* has been shown to have numerous single base substitutions and therefore fragments from *lpxA* may have been expected. However, only one *Sau*3A site was observed in the 790 bp *lpxA* sequence (Knibb, 2001), suggesting *Sau*3A fragments containing *lpxA* may have been too long to be detected by the current method. The apparent selectivity of the method applied in this study towards shorter fragments may explain why *lpxA* and fragments from other genes were not isolated.

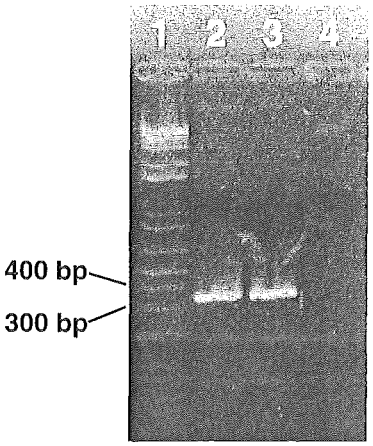


Figure 3.5 Amplicons generated from a PCR using the *flgE* primers 00-59 and 00-60. An anticipated 350 bp fragment was generated. Lane 1, 1 kb plus DNA ladder; lane 2, NCTC11168 DNA; lane 3, F38011 DNA; lane 4, negative control.

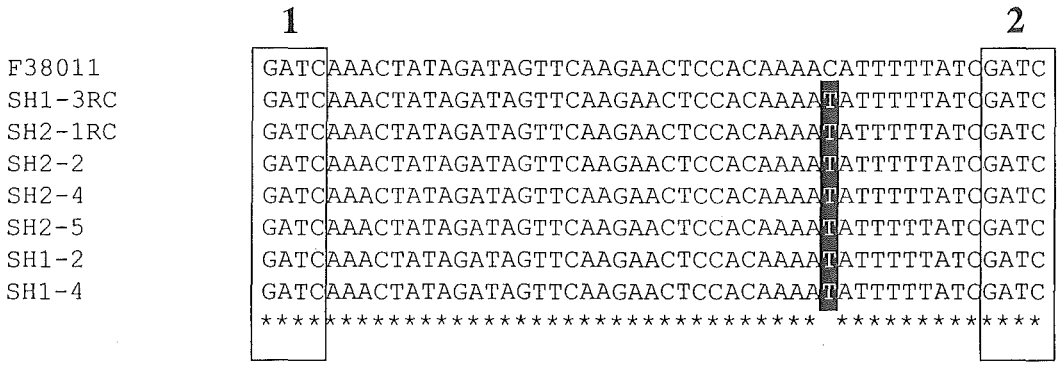


Figure 3.6 ClustalX alignment of F38011 with *flgE* fragments isolated from two subtractive hybridisations. The sequences for SH1-3 and SH2-1 were reversed and complemented (indicated by 'RC') in order to align with the other sequences. The boxes (1 and 2) indicate *Sau3*A restriction sites. The highlighted area indicates the single base pair difference between F38011 and the sequences isolated from NCTC11168.

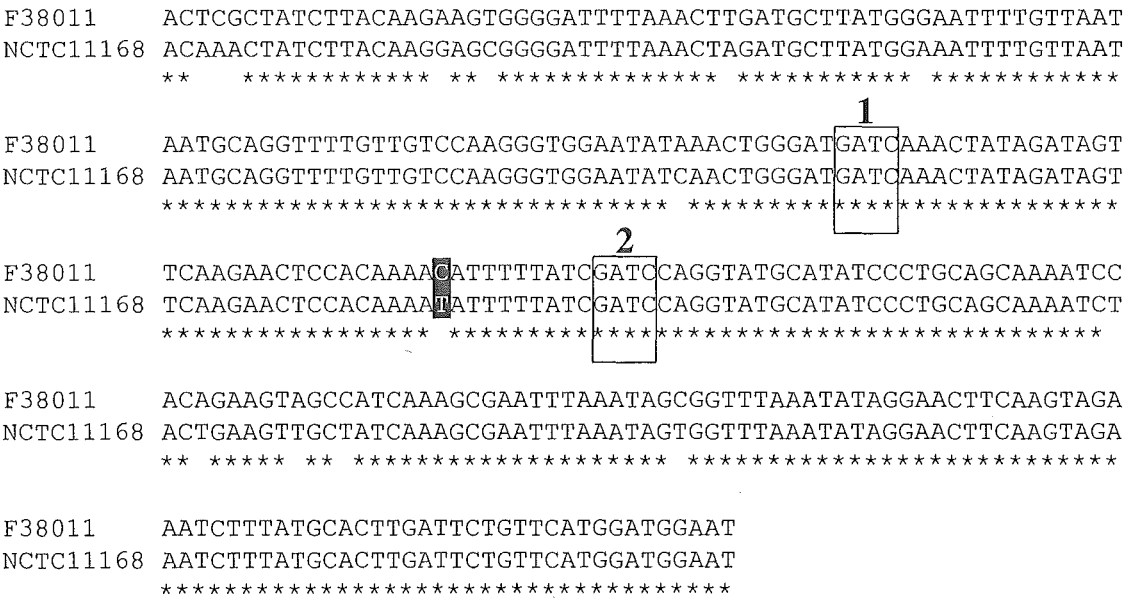


Figure 3.7 An alignment of the sequenced *flgE* amplicons from NCTC11168 and F38011. The boxed areas indicate *Sau3*A restriction sites (1 and 2). No * beneath the aligned bases denotes a sequence difference. The base substitution found in the fragments from the subtractive hybridisation is highlighted.

When the sequenced 350 bp amplicons from the *flgE* PCR of F38011 and NCTC11168 genomic DNA were aligned, 14 base substitutions were observed (Figure 3.7). The nucleotide variation between NCTC1168 and F38011 in the sequenced *flgE* region was 4%. This suggests *flgE* varies between isolates. The variation between the *flgE* genes of NCTC11168 and F38011 is higher than the 1% variation observed in the *lpxA* genes of these strains (Knibb, 2001). It is also higher than the 1.4% variation reported for *cadF* in strains of *C. jejuni* (Konkel, *et al.*, 1999b). Only two *Sau*3A restriction sites were observed within the generated sequences and these matched those of the isolated fragments. The importance of these results in terms of the pathogenic potential of *C. jejuni* cannot be determined from the work presented here.

Similar experiments would have been performed for the other fragments isolated, however time constraints did not allow this.

3.2 RAPD-PCR

Due to the lack of diverse fragments generated, RAPD-PCR was investigated as another means of isolating differences between strains with potential importance in virulence. In RAPD-PCR, profiles are generated by non-specific amplification with arbitrary primers. It may be possible that a band present in one profile that is absent in another relates to the presence or absence of a gene in the respective isolates. The aim of this experiment was to first determine if RAPD-PCR generated different profiles for F38011 and NCTC11168. If different profiles were generated, the questions would be asked of whether amplicons of the same size were the same, and whether amplicons that differed represented true differences between strains. RAPD profiles were first generated using a range and combinations of primers to amplify genomic DNA from NCTC11168 and F38011 respectively (Figure 3.8 and Figure 3.9). The profiles generated using the primers ERIC-1R and ERIC-2 were selected for further investigation as they were the same in each strain except for the presence of a 1 kb amplicon in F38011 that was absent from NCTC11168.

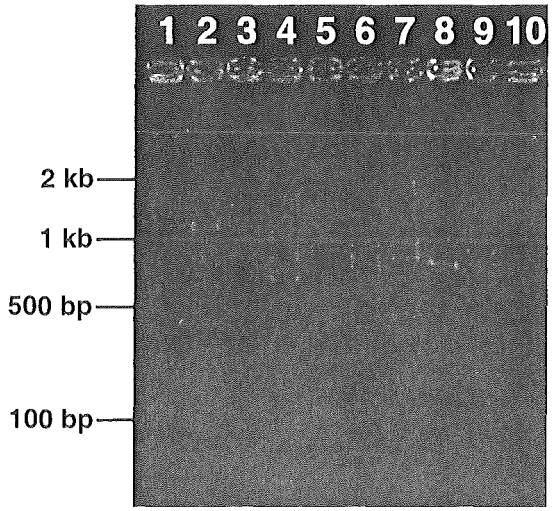


Figure 3.8 RAPD-PCR using a combination of primers to gain profiles from NCTC11168 (lanes 2, 4, 6 and 8) and F38011 (lanes 3, 5, 7 and 9). Lanes 1 and 10, 1 kb plus DNA ladder; lanes 2 and 3, NR2; lanes 4 and 5, ERIC -1R; lanes 6 and 7, ERIC-2; lanes 8 and 9, ERIC-1R and ERIC-2.

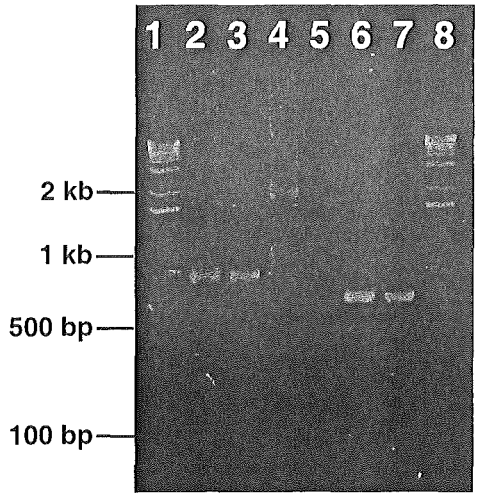


Figure 3.9 RAPD-PCR using a combination of primers to gain profiles from NCTC11168 (lanes 2, 4 and 6) and F38011 (lanes 3, 5 and 7). Lanes 1 and 8, 1 kb plus DNA ladder; lanes 2 and 3, REP1R-1; lanes 4 and 5, REP2-1; lanes 6 and 7, REP1R-1 and REP2-1.

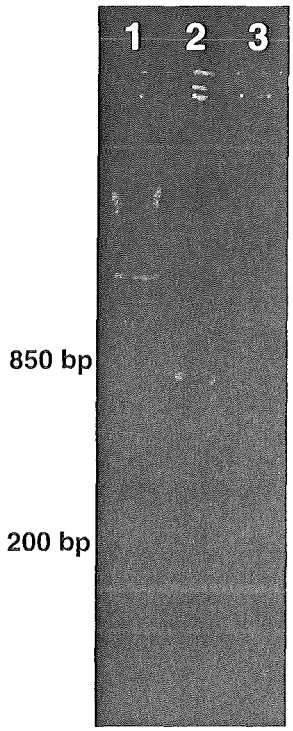


Figure 3.10 An example of variability of RAPD-PCR profiles. The profiles were generated using the primers ERIC-1R and ERIC-2 on NCTC11168 DNA (lane 2) and F38011 DNA (lane 3). The 1 kb plus DNA ladder is in lane 1. Note the absence of bands in lane 3, compared to Figure 3.8 (lane 9).

However, difficulties involving the reproducibility of the profiles were encountered. The stability of the profiles appeared very sensitive to slight changes in DNA concentration used in the reaction (Figure 3.10). The ERIC-1R/ERIC-2 profiles shown in Figure 3.10 (lane three) are considerably different to the profiles seen previously in Figure 3.8 (lanes eight and nine). Unfortunately, time constraints did not allow for these problems to be resolved. The irreproducibilty of the profiles would have meant that any results obtained from continued analysis would be unsubstantiated. For these reasons, RAPD-PCR was disregarded as a reliable means of isolating differences between strains.

3.3 Differential gene expression

Detection of differential expression of genes in strains of *C. jejuni* was initially investigated as a means for detecting gross differences between two strains. This approach required reliable isolation of RNA and reverse transcription of mRNA. Suitable controls to rule out the possibility of the generation of false positives due to contamination with residual DNA were also necessary. Differential gene expression may be detected between the same strain under different conditions or different strains under the same condition. In order to assess the application of such techniques, two control experiments were devised using two isogenic sets already available in the laboratory.

The isogenic sets used in this study were F38011*ΔciaB* and F38011, and F38011*ΔgmhD* and F38011. The gene *ciaB* encodes a cell internalisation factor (Konkel *et al.*, 1999a) and is induced by conditions of the human gut. When a mRNA-based subtractive hybridisation is performed between F38011 and F38011*ciaBΔ*, induced by bile salts, the resulting products should correspond to the *ciaB* gene. The *gmhD* gene is involved in heptose synthesis and is constitutively expressed. A subtractive hybridisation between F38011 and F38011*gmhDΔ* should only result in the isolation of the *gmhD* gene.

3.3.1 Experimental controls

An assurance of the absence of DNA is required in order to validate RT-PCR results. For this reason, a number of controls were implemented to ensure only RNA was reverse transcribed and detected in subsequent PCR. Isolated RNA was subjected to digestion with DNaseI to remove potential contaminating DNA from the sample. Figure 3.11 demonstrates the controls performed for a 23S rDNA PCR on reverse transcribed RNA from a culture grown under standard conditions and a culture grown in the presence of DCA. After treatment with RNase, contaminating DNA in the RNA sample can be amplified to yield a PCR product of the expected size. The presence of an amplicon in lane six but not in lane three indicates that contaminating DNA may be a greater problem in some samples than in others. Samples which had been both DNase- and RNase-digested failed to give an amplicon, indicating there was no nucleic acid remaining in the sample and DNase digestion had gone to completion. The RT negative control (lane eight) also indicates complete DNase digestion of the sample. The results from the controls that were implemented indicate that the RT reaction was specific for mRNA and subsequent PCR amplification was not due to contaminating DNA.

3.3.2 Induction of *ciaB*

Transcription of the gene *ciaB* is necessary for invasion of host epithelial cells (Konkel *et al.*, 1999a) and should be induced in the presence of the bile salt deoxycholic acid (DCA). RNA was isolated from F38011 grown under standard conditions and F38011 grown in the presence of 0.1% DCA. If *ciaB* was induced by bile salt, the expected result would be PCR detection of *ciaB* cDNA only from the induced culture. The results shown in Figure 3.12 indicate *ciaB* was not expressed under the conditions provided, due to the absence of the expected 500 bp amplicon from cDNA synthesised from the culture grown in the presence of DCA. Only contaminating DNA was amplified from the RNase-digested controls (Figure 3.12, lanes three and six). Similar results were also obtained for the HS:19 expressing strain KLC4315 grown under standard conditions and in the presence of DCA (data not shown).

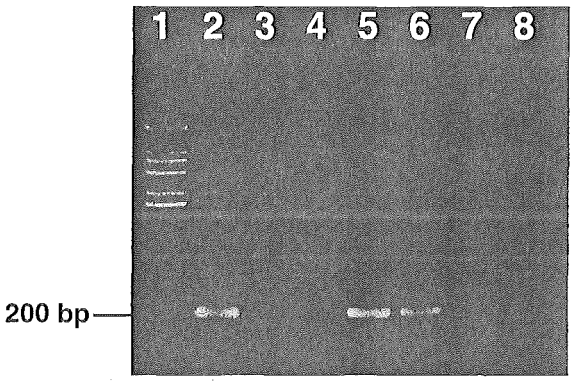


Figure 3.11 Products from a 23S rDNA PCR performed on cDNA and controls from F38011 grown under standard conditions (lanes 2, 3, 4 and 8) and F38011 grown in the presence of DCA (lanes 5, 6, and 7). Lane 1, 1kb plus DNA ladder; lane 2 and 5, DNaseI-digested; lanes 3 and 6, RNaseA-digested; lanes 4 and 7, RNaseA- and DNaseI-digested; lane 8, DNaseI-digested RNA but not reverse-transcribed.

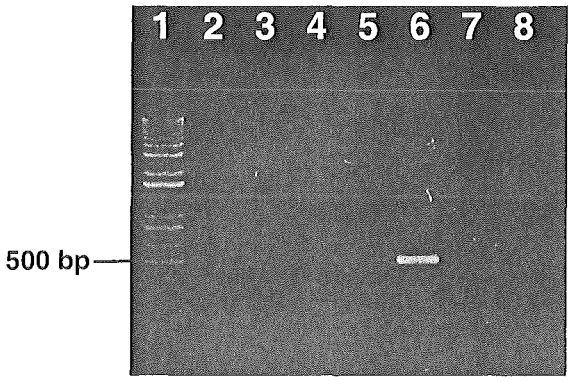


Figure 3.12 Products from a *ciaB* PCR performed on cDNA from F38011 grown under standard conditions (lanes 2, 3, and 4) and F38011 grown in the presence of DCA (lanes 5, 6, 7 and 8). Lane 1, 1 kb plus DNA ladder; lanes 2 and 5, DNaseI-digested; lanes 3 and 6, RNaseA-digested; lanes 4 and 7, RNaseA- and DNaseI-digested; lane 8, DNaseI-digested RNA but not reverse transcribed.

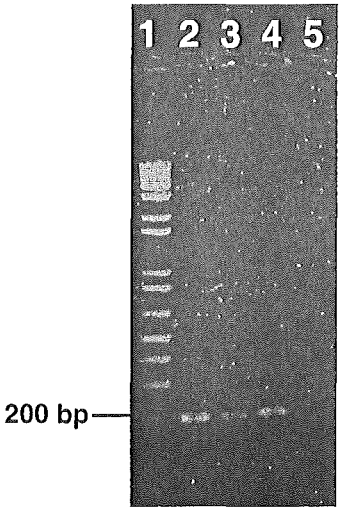


Figure 3.13 Products from a *hupB* PCR performed on cDNA from F38011 grown under standard conditions (lanes 2 and 3) and F38011 grown in the presence of DCA (Lane 4). Lane 1, 1 kb plus DNA ladder; lane 2, F38011 DNA (positive control); lanes 3, F38011 cDNA (DNaseI-treated); lane 4, cDNA from induced F38011 (DNaseI-treated); lane 5, negative control for PCR.

The gene *hupB* is located immediately downstream from *ciaB* and is constitutively expressed (Konkel *et al.,* 1999a). Therefore, *hupB* should be detected in both the induced and uninduced cultures. PCR amplification of *hupB* was detected in F38011 grown under standard conditions and F38011 grown in the presence of DCA (Figure 3.13). This PCR was performed to determine that the lack of detection of *ciaB* in the culture grown with DCA was indeed due to lack of induction in this condition and not to other factors such as poor mRNA integrity and reverse transcription.

3.3.3 Detection of *gmhD* expression

A PCR using primers 97-01 and 97-02 to detect *gmhA* was performed. No amplification would be expected from F38011*ΔgmhD* cDNA as the genes in this heptose synthesis region are expressed as a single polycistronic message and *gmhD* is upstream from *gmhA*. Therefore, an insertional disruption in *gmhD* will prevent transcription of *gmhC/D* and *gmhA*, downstream (Klena, J. D., & Vasan, D., unpublished results). The results in Figure 3.14 shows *gmhA* from F38011*ΔgmhD* was not amplified with these primers as expected. However, no amplicon was detected from F38011 cDNA. Primers 99-25 and 99-26 generated a positive amplicon of 650 bp in size for *gmhD* from F38011 DNA but no amplification was observed from F38011 cDNA (Figure 3.15).

In order to determine if the lack of detection of *gmhD* expression was due to potential problems with the primers used, a PCR to detect *lpxA* expression was also performed. *lpxA* encodes a protein involved in lipid A synthesis (Ibbitt, 1997). This PCR generated the expected 800 bp fragment in the DNA controls, but failed to amplify both F38011 and F38011*ΔgmhD* cDNA (Figure 3.16). These results indicate that the lack of detection of *gmhD* expression may be due to problems with the RNA isolation or the RT reaction.

To help elucidate the source of the low detection problems, DNaseI-treated RNA and cDNA was obtained from an independent source, ESR. 23S rDNA and *lpxA* PCR were performed on this cDNA and cDNA synthesised from the RNA. All samples positively

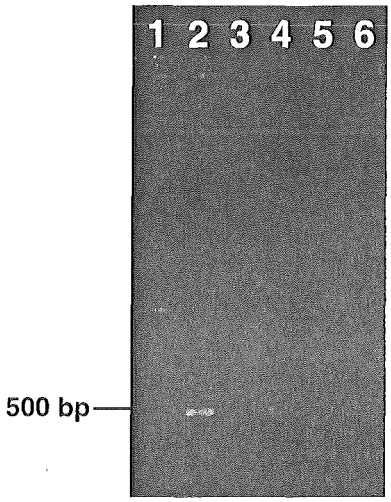


Figure 3.14 Products from a *gmhA* PCR using the primers 97-01 and 97-02. Lane 1,1 kb plus DNA ladder; lane 2, F38011 DNA; lane 3, F38011 cDNA; lane 4, F38011Δ*gmhD* DNA; lane 5, F38011Δ*gmhD* cDNA; lane 6, negative control.

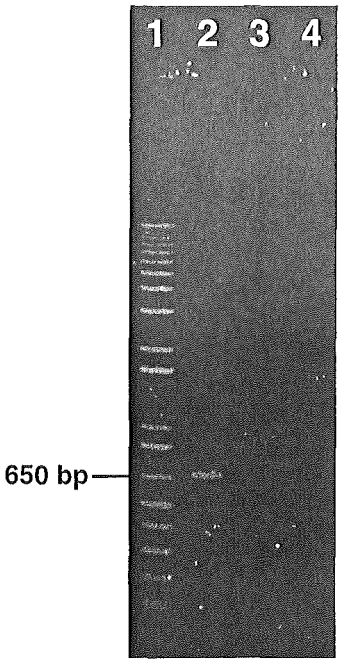


Figure 3.15 Products from a PCR using *gmhD* primers 99-25 and 99-26. Lane 1, 1 kb plus DNA ladder; lane 2, F38011 DNA (positive control); lane 3, F38011 cDNA; lane 4, no DNA (negative control).

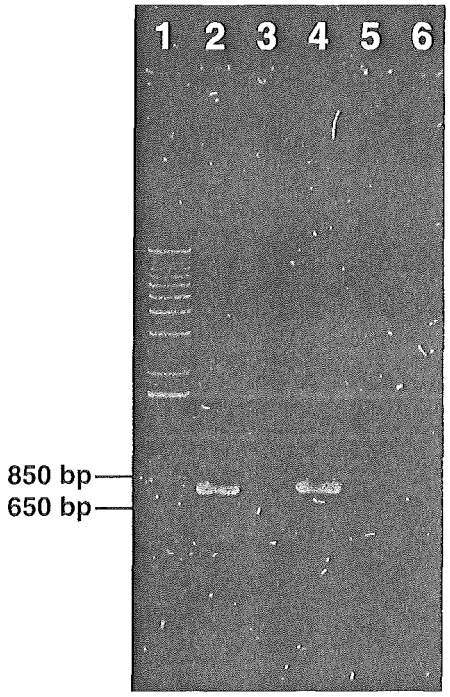


Figure 3.16 Products from a *lpxA* PCR performed on cDNA from F38011 and F38011Δ*gmhD* using primers 96-24 and 96-25. Lane 1, 1 kb plus DNA ladder; lane 2, F38011 DNA (positive control); lane 3, F38011 cDNA; lane 4, F38011Δ*gmhD* DNA (positive control); lane 5, F38011Δ*gmhD* cDNA; lane 6, no DNA (negative control).

amplified for 23S rDNA (Figure 3.17) but only the induced F38011 cDNA sample from ESR amplified *lpxA* and only weakly (Figure 3.18). These results suggest that the lack of detection may be related to low transcript abundance in the sample. 23S rDNA amplification from cDNA has been positive each time (Figure 3.11 and Figure 3.17). Due to the presence of multiple copies of this gene in the genome at any one time, a higher abundance of 23S rDNA transcripts available for RT in a sample may be expected.

All the above experiments were repeated to ensure the reproducibility of the results obtained. Similar results were obtained each time (data not shown). Due to the problems incurred with the RNA approach and the time constraints of a Master's project, this line of work was discontinued in favour of the DNA approach presented earlier.

3.4 Real-time TaqMan® analysis

TaqMan® PCR (5´-nuclease PCR) is a more sensitive detection method than conventional PCR and can allow for a quantitative assessment of mRNA expression levels. TaqMan® PCR was performed on cDNA from RNA samples to aid interpretation of the negative results obtained in the above sections. Unlike conventional PCR, where only the end product amplification may be observed by gel electrophoresis, TaqMan® PCR generates a fluorescent signal which is detected throughout the course of the reaction. The fluorescent signal is proportional to the amount of PCR product (Qaigen News, 2001). A normalised reporter ratio (R_n) is obtained for each reaction. This refers to the fluorescent intensity of a reporter dye, divided by the fluorescent intensity of a passive reference. A threshold R_n was arbitrarily selected for the run, based on the steepest point of the logorithmic curve generated by the change in R_n against PCR cycle (Gilpin, 2001). The threshold cycle (C_T) refers to the cycle at which the R_n of the reaction exceeds the threshold that was selected. Every three cycles of PCR amplification represents an approximately 10-fold reduction in expression, provided the RT products are an accurate representation of the mRNA in the

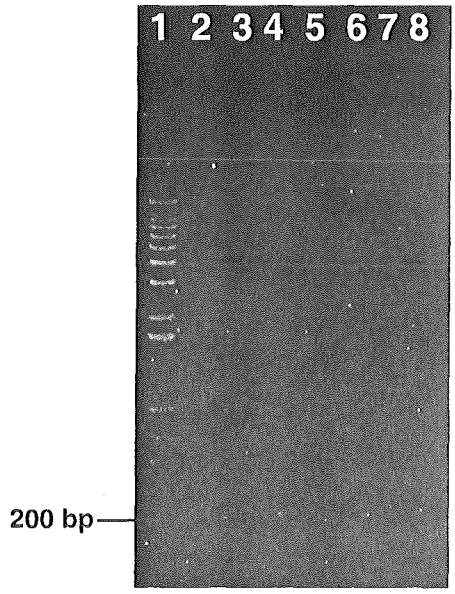


Figure 3.17 Products from a 23S rDNA PCR performed using cDNA synthesised from DNaseI-digested RNA. The RNA was extracted using either the RNeasy kit or the Trizol RNA extraction method. RNA samples for lanes 6 and 7 were provided by ESR. Lane 1, 1 kb plus DNA ladder; lane 2, F38011 DNA; lane 3, F38011 cDNA - trizol; lane 4, F38011 cDNA - RNeasy; lane 5, F38011Δ*gmhD* - trizol; lane 6, F38011 cDNA - trizol; lane 7, induced F38011 cDNA - trizol; lane 8, negative control for PCR.

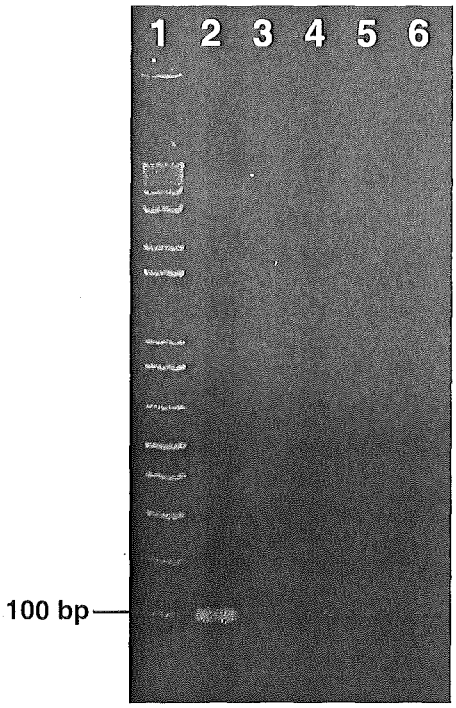


Figure 3.18 Products from a *lpxA* PCR using the multiplex primers, resolved on a 2% agarose gel. Lane 1, 1 kb plus DNA ladder; lane 2, F38011 DNA; lane 3, F38011 cDNA - ESR; lane 4, induced F38011 cDNA - ESR; lane 5, F38011 cDNA; lane 6, negative control.

original sample (Gilpin, 2001). Therefore, a high C_T indicates little or no expression. The progamme used for the TaqMan® PCR ran for 60 cycles.

Figure 3.19 shows the lowest C_T values were obtained for 23S rDNA. This indicates 23S rDNA had the greatest expression of genes targeted in this experiment. This would be expected due to the potential for multiple copies of 23S rDNA in the genome to be expressed.

Figure 3.19 indicates that no expression of *ciaB* was detected for F38011 when grown under standard conditions (without DCA) (C_T equals cycle 60). This result was expected as *ciaB* requires induction by bile salt. The C_T obtained for F38011 (0.1% DCA) was 38 for *ciaB*, indicating some expression of *ciaB* can be detected and *ciaB* can be induced by 0.1% DCA. However, a similar result was obtained for F38011Δ*ciaB* (0.1% DCA), where no detection of *ciaB* was expected. This result suggests, either F38011Δ*ciaB* has lost the disruption in the *ciaB* gene and transcription of *ciaB* has resumed, or more likely, incomplete digestion with DNaseI has led to DNA contamination of the PCR. It may also be possible that a truncated *ciaB* transcript has been detected from F38011Δ*ciaB*. Due to the nature of the insertional mutation in this strain, an intact promoter is still linked to the 5´ region of *ciaB*. Taqman® PCR detection in of *ciaB* from F38011Δ*ciaB* would be dependent on the position of the primers and the probe, relative to this 5´ region. The presence of the insertion in F38011Δ*ciaB* was confirmed in Section 3.1.1. The C_T of 29 for *ciaB* obtained from F38011Δ*gmhD* also suggests DNA contamination of the sample. Induction of *ciaB* was not expected in any strain grown under standard conditions. The comparative C_T values obtained for *ciaB* in each of the samples indicates DNA contamination may be a problem in some samples but not in others. However, due to the potential for DNA contamination, shown in Figure 3.19, it cannot be inferred from the results that *ciaB* was successfully induced by 0.1% DCA. The low levels of expression and/or DNA contamination would not have been detected by the conventional PCR and gel electrophoresis performed in the earlier sections. This is because amplification was only permitted to occur for 30 cycles, and C_T values presented in Figure 3.19 were above this number of cycles.

The relatively high C_T values obtained from the TaqMan® PCR of *lpxA* support the lack of detection observed from cDNA by conventional PCR (Figures 3.14 and 3.16) for the same reasons stated above.

Unfortunately, no probe was available to detect *gmhD* by TaqMan® PCR. However, the similarity between results obtained for F38011Δ*gmhD,* and those obtained for the other samples tested in this experiment indicate a C_T value above the number of cycles performed in a conventional PCR would likely have been obtained.

A positive DNA control and a negative control (no nucleic acid) were included in the TaqMan® PCR. The positive control gave C_T values of 17, 31 and 42 for 23S rDNA, *ciaB* and *lpxA*, respectively (data not shown). The negative control had not been detected by the TaqMan® PCR, using any of the primers/probes, at cycle 60 (C_T of 60) (data not shown).

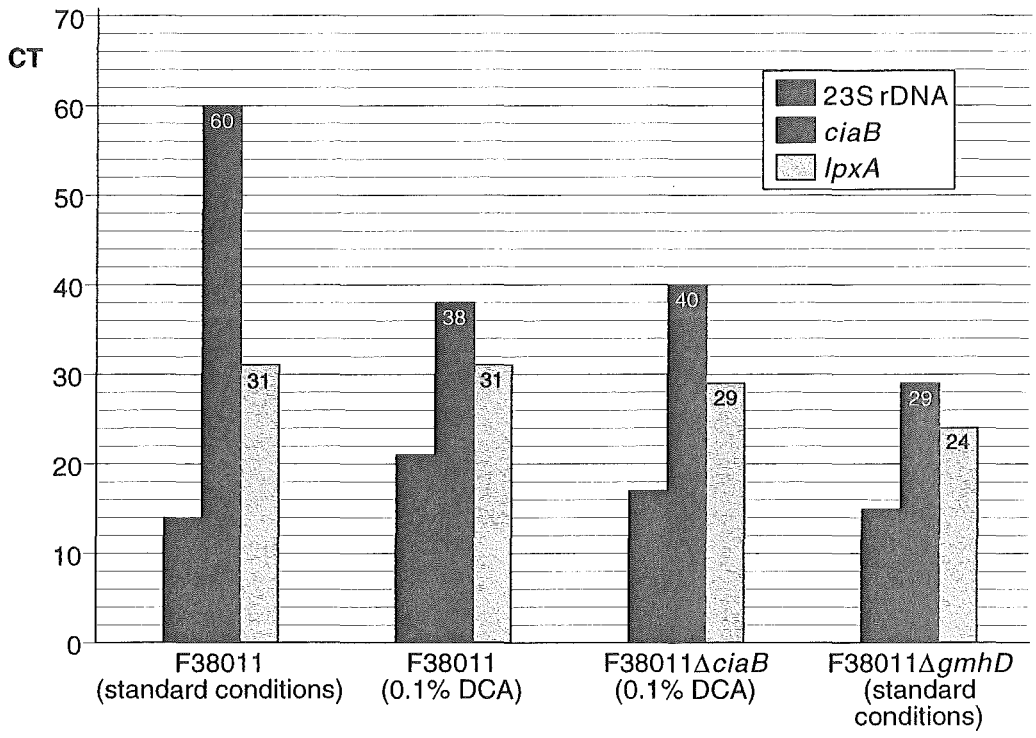


Figure 3.19 CT values obtained from real-time TaqMan PCR on cDNA from F38011 grown under standard conditions, F38011 and F38011Δ*ciaB* grown with 0.1% DCA, and F38011Δ*gmhD* grown under standard conditions. The genes targeted are indicated in the legend box.

Chapter 4

Discussion

In recent years, *C. jejuni* has emerged as an important human enteric pathogen, causing significant morbidity and representing a significant burden on healthcare systems and loss of human productivity. *C. jejuni* appears ubiquitous in the environment and may be isolated from a number of animal and environmental sources. It is unclear whether all strains of *C. jejuni* cause disease in humans, or whether this property is ubiquitous. Epidemiological studies have applied various identification methods in order to determine the similarities between isolates from different reservoirs (Aeschbacher & Piffaretti, 1989; Korolik *et al.,* 1995; Duim *et al.,* 1999; Dingle *et al.,* 2001). There is some evidence to suggest there may not be a complete overlap in clinical isolates and environmental isolates. Determination of the pathogenic potential of strains from certain sources is important in order to assess the actual risk posed to human health by *C. jejuni.*

A key factor in an investigation of pathogenic potential is understanding possible virulence genes or mechanisms that may be involved in the disease process. In comparison to other bacterial pathogens, little is understood of the mechanisms involved in disease caused by *C. jejuni.* Until very recently, random mutagenesis approaches have not been available as a means of studying potential virulence factors in *C. jejuni.* Therefore, an investigation of the application of other techniques available for the study of pathogenicity was necessary. DNA-based subtractive hybridisation was selected as one technique providing the potential to find genes that might be important in virulence, by detecting intra-species variation. The application of RNA-based methodologies and RAPD-PCR in the study of pathogenicity were also investigated. The application of these techniques in the study of *C. jejuni* is discussed below.

4.1 Detection of strain differences in *C. jejuni*

The control subtractive hybridisation experiment (Section 3.1.1) between the isogenic strains F38011Δ*ciaB* (tester) and F38011 (driver) was expected to isolate the kanamycin resistance gene that was inserted in F38011Δ*ciaB* when the insertional disruption of the *ciaB* gene was constructed (Konkel *et al.*, 1999a). This experiment was performed to assess the ability of the PCR-based subtractive hybridisation method (Akopyants *et al.*, 1998) to isolate a known difference between strains. The presence of kanamycin resistance gene fragments in the products from the subtractive hybridisation was not detected by Southern hybridisation. This result suggests that the kanamycin resistance gene was not isolated by the subtractive hybridisation. The subtractive hybridisation was found to favour the isolation of small fragments. Thus, large fragments may have been lost during the course of the experiment. However, the probe that was used in the Southern hybridisation was large (2.3 kb) and the fragments to be detected, ie., fragments of the kanamycin resistance gene, could have potentially been small. This discrepancy between the size of the probe and the fragment size may have inhibited probe-binding. Therefore, the results from this experiment were inconclusive.

To further test the application of the DNA-based subtractive hybridisation method to isolate differences between strains, subtractive hybridisation between NCTC11168 (tester) and F38011 (driver) was performed. From two consecutive and identical subtractive hybridisations, a total of eleven isolated fragments were sequenced. Blastx searches and sequence alignments revealed that seven of the sequences were identical and had significant identity with the gene encoding FlgE. Sequencing of the *flgE* gene from NCTC11168 and F38011 indicated that these isolated 49 bp fragments had one base-pair substitution when aligned with the F38011 *flgE* sequence. Addtionally, three identical fragments, cloned independently, had significant identity with an A/G-specific adenine glycosylase and one fragment had significant identity with an L-serine dehydratase. All sequences isolated by the subtractive hybridisation had 100% identity with the NCTC11168 genome sequence, indicating specific isolation of fragments from the tester strain. The sequences obtained

from the subtractive hybridisation experiments demonstrate the reproducibility of this technique. Isolation of genes encoding an A/G-specific adenine glycosylase and an L-serine dehydatase was not expected, due to their essential roles in DNA-repair and general cell functions. It would be expected that these genes would be largely conserved between strains and therefore would not be detected by subtractive hybridisation.

The original PCR-based subtractive hybridisation method applied by Akopyants *et al.* (1998) was shown to be most successful when tested on isogenic *H. pylori* strains that differed only in the presence or absence of the 37 kb *cag* PAI, which is equivalent to about 2% of the *H. pylori* genome. This difference is considerably larger than the kanamycin resistance gene present in F38011Δ*ciaB* and absent from F38011, which is 1.2 kb (Trieu-Cust *et al.,* 1989). A hybridisation experiment by Akopyants *et al.* (1998) showed more than 90% of clones had derived from the *cag* PAI. To further test their method, they applied subtractive hybridisation to two unrelated strains of *H. pylori.* They reported that the recovery of strain-specific sequences was lower than that with the isogenic control strains. Only about 50% of clones were judged to be specific to the tester strain by hybridisation and the sequences of 17 of the 18 clones tested were either absent or substantially different from sequences in the driver strain. The results from the sequenced *flgE* fragments in this study showed a substantial amount of sequence identity with the driver. It may have been that the two strains, NCTC11168 and F38011, were not dissimilar enough to effectively isolate differences between them. *H. pylori* is a highly recombinatory species, more so than *C. jejuni* (Achtman *et al.,* 1999; Suerbaum *et al.,* 2001), and the strains used in the study by Akopyants *et al.* (1998) were isolated from very distinct sources: a human gastric ulcer and a rhesus monkey.

4.2 Sequences isolated by DNA-based subtractive hybridisation

4.2.1 FlgE

The results from the subtractive hybridisation between NCTC11168 and F38011 showed that seven of the eleven fragments that were sequenced shared significant identities with the flagellar hook protein FlgE of *C. jejuni* (Section 3.1.3). The flagellar hook connects the filament to the basal body and functions as a joint to transmit the rotation of the rod of the basal body to the filament (Lüneberg *et al.,* 1998). The result obtained in this study, indicating a difference between strains in FlgE, is not unexpected. The central, surface-exposed region of FlgE has shown hypervariability (less than 10% identity in the amino acid sequences) among strains of *C. jejuni* (Lüneberg *et al.,* 1998). Semivariable (greater than 20% identity) and highly variable (10 to 20% identity) regions have also been observed (Lüneberg *et al.,* 1998). Sequence divergence in *flgE* genes from *C. jejuni* and *C. coli* has been observed, although the gene size and 5′ and 3′ DNA sequences appear conserved (Kinsella *et el.,* 1997).

Kinsella *et al.* (1997) showed that mutants of *C. coli* defective in hook production were nonmotile and lacked flagellar filaments. Analyses of *flgE* mutants indicated that the carboxy terminus of FlgE was necessary for assembly of the hook structure and the N-terminal region was required for its secretion (Kinsella *et al.,* 1997). Lack of *flgE* expression has been found not to result in repression of flagellin expression (Kinsella *et al.,* 1997). In this study, the *flgE* fragments isolated by subtractive hybridisation fall within the N-terminal region close to a semivariable region (Lüneberg *et al.,* 1998).

A study into the antigenic properties of the *Campylobacter* flagellar hook protein demonstrated the first reported instance of an epitope shared between flagellin and hook proteins (Power *et al.,* 1992). Antigenic variability in hook proteins isolated from different strains of *C. jejuni* has been suggested from the binding specificity of monoclonal antibodies raised against *C. jejuni* 5226, which bound exclusively to that strain (Glenn-Calvo *et al.,* 1994). This is supported by binding of monoclonal antibodies to the intact hook of *C. jejuni* 5226, as observed by immunoelectron microscopy (Glenn-Calvo *et al.,* 1994), and

that the variable and hypervariable domains of FlgE are likely to be exposed at the surface (Lüneberg *et al.,* 1998). It has been suggested that *C. jejuni* may respond to the selective pressure of its hosts by altering surface-exposed antigenic determinants and antigenic variation may provide a selective advantage, especially in areas where *C. jejuni* is endemic and reinfections of hosts occur frequently (Lüneberg *et al.,* 1998). The ability to colonise dynamic intestinal environments appears to be the current favoured function inferred from rapid variation of surface antigens, rather than immune avoidance (Parkhill *et al.,* 2000; Hänninen *et al.,* 2001).

The sequences corresponding to FlgE highlight the potential of subtractive hybridisation to detect differences between strains. Certainly, isolation of such sequences would have been expected, due to the known variability of this region (Lüneberg *et al.,* 1998). The role of FlgE in motility, an essential virulence determinant in *C. jejuni,* also lends support to the use of this technique to search for potential virulence genes.

4.2.2 A/G-specific adenine glycosylase

The results from Blastx alignments of the sequenced fragments SH1-1, SH1-5, and SH2-6, indicated significant identity with a *C. jejuni* A/G-specific adenine glycosylase (Table 3.1). In *H. pylori* and *E. coli*, an A/G-specific adenine glycosylase is encoded by *mutY* (Achtman *et al.,* 1999). In *E. coli* MutY is involved in protection of cells from the mutagenic effects of oxidative damage of guanine to 7,8-dihydro-8-oxoguanine (8-oxoG) (Gifford *et al.,* 2000). As a base excision repair enzyme, MutY removes A in 8-oxoG-A mispairs and failure of this process results in a GC→TA transversion (Chepanoske *et al.,* 1999; Gifford *et al.,* 2000). Enzymes involved in base excision repair are highly conserved between species, and eukaryotic homologues of *E. coli* MutY have been identified (Chepanoske *et al.,* 1999). The properties of substrate recognition and repair by MutY are likely to be shared with the entire class of base excision repair glycosylases, including those in higher organisms (Chepanoske *et al.,* 1999). Due to the integral role of an A/G-specific adenine

glycosylase in DNA repair in *C. jejuni*, it would not be an expected product of a subtractive hybridisation between NCTC11168 and F38011.

Sequencing of the putative *mutY* from F38011 and NCTC11168 was not performed, as it was for *flgE*. This would have enabled the elucidation of any base changes in this gene between the two strains. A MLST study of *H. pylori* used sequences from the *mutY* locus (Achtman *et al.*, 1999). This study showed little non-synonymous variation (changes in amino acid sequence) in the loci sequenced, but all sequences showed high levels of synonymous sequence variation. In light of this information from *H. pylori* MLST data, sequencing of *mutY* from *C. jejuni* F38011 and NCTC11168 might have shown silent mutations that would not affect protein structure. The *flgE* results showed reproducible isolation of sequences with just one base substitution. Therefore, it may be reasonable to assume that isolation of a *mutY* fragment in the subtractive hybridisation was not a false positive result. However, without sequencing of *mutY* from F38011 it is impossible to state this conclusively.

4.2.3 L-serine dehydratase

Results from the Blastx search for the sequenced fragment SH2-3, indicated 100% identity with a *C. jejuni* NCTC11168 L-serine dehydratase, encoded by the gene *sdaA* (Table 3.1). L-serine dehydratases catalyse the irreversible overall deamination of L-serine to pyruvate (Hofmeister *et al.*, 1997). Like *mutY*, the essential role of the SdaA protein in cell biochemical functions, suggests that *sdaA* would be relatively conserved and therefore not an expected product of the subtractive hybridisation procedure. SdaA would also not be expected to be exposed to the selection pressures of the host immue system. Sequencing of *sdaA* was not performed, but silent mutations may have been present. Sequencing of *sdaA* would have to be performed to confirm that the isolation of *sdaA* by subtractive hybridisation was not due to the generation of a false positive sequences.

4.3 Optimisation of DNA-based subtractive hybridisation

The results presented in Section 3.1 indicate that for the successful application of a DNA-based subtractive hybridisation, optimisation of the protocol be would required. Many steps are involved in the subtractive hybridisation protocol. Some or all of these may need to be optimised for future applications. The limited time allotted for these experiments, due to the time invested in RT-PCR detection methods, did not permit full optimisation of the protocol. Aspects of the protocol that may have been tested or improved are discussed below.

4.3.1 DNA purity

The guanidium thiocyanate DNA isolation protocol (Section 2.4.1) was used in this study. It was a rapid and reliable method for DNA isolation and DNA isolated in this way was deemed suitable for subsequent use in PCR. However, there may still be residual protein and carbohydrate contamination remaining in the sample. DNA preparations used in this study gave an A_{260}/A_{280} ratio of approximately 2.1. The purity of the DNA used in the subtractive hybridisation may have affected the outcome of the subtractive hybridisation in a number of ways.

Protein and carbohydrate contamination of the sample may have inhibited restriction endonuclease digestion, thereby increasing the yield of longer, undigested DNA fragments present in the sample. This in turn may have altered the hybridisation dynamics. The contamination itself may also have inhibited hybridisation. Long DNA fragments may form complex networks that prevent the formation of the appropriate hybrids needed to position the two independent adaptors (adaptor 1 and adaptor 2) at the ends of the target molecules (Diatchenko *et al.*, 1999). Longer fragments may also be excluded from PCR amplification due to the preferential amplification of shorter fragments (Straus & Ausubel, 1990; Akopyants *et al.*, 1998; Diatchenko *et al.*, 1999). Contamination in the sample may have resulted in DNase digestion of some of the sample DNA, leading to short fragments

that may have been preferentially amplified in subsequent PCR. Straus and Ausubel (1990) suggested DNA for use in subtractive hybridisation should be free from biological contaminants and therefore used the CTAB DNA isolation protocol, followed by two phenol:chlorofrom extractions.

4.3.2 DNA concentration

The concentration of DNA used in this study was measured spectrophotometrically. This method of concentration determination may not have been sufficiently accurate for application in subtractive hybridisation (Straus & Ausubel, 1990). Calculation of DNA concentration by gel electrophoresis and comparison of band intensities to λ DNA of known concentrations may have been more accurate (Straus & Ausubel, 1990). Inaccurate calculation of DNA concentration may have influenced the tester:driver ratios, thereby altering the hybridisation stringency. If the tester:driver ratio was unintentionally lowered due to inaccurate determination of DNA concentration, a lower hybridisation stringency may have resulted. Consequently, a higher rate of false positives may have resulted (Hubank & Schatz, 1999).

4.3.3 Ligation of adaptors

The ligation of the adaptors to the ends of tester sequences is an important step in the subtractive hybridisation. This step allows PCR amplification and therefore kinetic enrichment of target sequences. In this study ligation efficiency was not assessed until the subtractive hybridisation was completed and the sequences from the isolated fragments were analysed. The sequenced fragments contained primer sequences for NP1 and NP2, indicating that adaptor ligation had been successful. NP1 and NP2 match the 3′ ends of adaptor 1 and adaptor 2, respectively, and were used for the final nested PCR. The presence of NP1 and NP2 sequences bordering the isolated sequences suggest that the adaptors must have ligated to the original restriction fragment.

Ligation efficiency could have been tested using the primer P1 and a gene-specific primer (Diatchenko *et al*, 1999). It would have been necessary for the amplified gene-specific fragment to contain no *Sau*3A restriction site and assays would had to have been performed to ascertain the most suited gene-specific primer (Diatchenko *et al*, 1999). A ligation efficiency of less than 25% would have substantially reduced the subsequent subtraction efficiency (Diatchenko *et al*, 1999). As ligation efficiency was not tested in this study, it would be impossible to judge whether this parameter had an impact on the subtraction hybridisations performed here. This would certainly be an important consideration for future applications of this method.

4.3.4 Hybridisation conditions

The first hybridisation took place at 65°C for 1.5 h and the second hybridisation was at 65°C, overnight (Akopyants *et al.* 1998). The tester:driver mass ratio also influences the hybridisation stringency (Hubank & Schatz, 1999). That the *flgE* sequences were isolated by the subtractive hybridisation between NCTC11168 and F38011, although only a single base change was observed in the sequences from the respective strains, could be indicative of a low hybridisation stringency. A low hybridisation stringency would be expected to yield fragments with sections of sequences matching those in the driver strain. However, the subtraction hybridisation between F38011 and F38011Δ*ciaB* did not yield the expected kanamycin cassette, which may suggest the hybridisation stringency was too high. A restriction fragment containing part of the kanamycin cassette could also contain part of the *ciaB* gene, and therefore could be removed under high stringency conditions.

High stringency conditions should exclude all sequences that are common between the tester and the driver. Figure 3.1 shows the subtractive hybridisation product of F38011Δ*ciaB* (tester) and F38011 (driver). If this product does not contain the kanamycin insert, as suggested by Southern hybridisation, then there must have been a high rate of generation of false positives, which is indicative of low stringency conditions.

The short length of the fragments observed, suggest these may be an artefact of PCR (Lisitsyn *et al.*, 1993; Diatchenko *et al.*, 1999). Short fragment length in the final product may also result from the more efficient hybridisation of shorter fragments (Diatchenko *et al.*, 1999). The efficiency at which driver sequences titrate homologous tester sequences may also have been lowered by restriction fragment length differences between tester and driver DNA (Akopyants *et al.*, 1998). In future applications of subtractive hybridisation, the optimal hybridisation conditions for strains of *C. jejuni* would have to be determined.

The hybridisation conditions may have been optimised in two ways. Firstly, the mass ratio of tester to driver DNA could have been increased. Increasing this ratio may ensure the tester molecules re-anneal to the excess of the driver and are removed, since the heteroduplex they form with the driver will not be amplified (Lisitsyn *et al.*, 1993). This may have improved primary enrichment for unique sequences present in the tester strain. Secondly, the hybridisation temperature could possibly be lowered. This may increase the favourable annealing of longer fragments in the pool DNA, perhaps limiting the overpopulation of short fragments that were observed.

4.3.5 PCR conditions

The fragments isolated by the subtractive hybridisation were very short, 49 to 67 bp in length. Small inserts in the final subtracted DNA library have been reported to be a typical drawback of the SSH technique applied in this study (Akopyants *et al.*, 1998; Diatchenko *et al.*, 1999). The combination of adaptors and the primer P1 used in this study are reported to introduce a weak suppressive PCR effect in the primary amplification because the complementary section of the adaptors is equal to the length of P1 (Akopyants *et al.*, 1998; Diatchenko *et al.*, 1999). It is suggested that under this condition, the amplification of short DNA fragments is significantly reduced and the risk of non-specific amplification also decreases (Diatchenko *et al.*, 1999). However, the results obtained in this study indicate an overabundance of short DNA fragments in the final product, despite the use of this

combination of adaptors and primers. The most likely explanation would appear to be preferential amplification of short fragments (Straus & Ausubel, 1990; Diatchenko *et al.,* 1999).

The number of cycles used in the secondary PCR may also have influenced the outcome of the final products observed. In this study, 25 cycles of PCR amplification were performed in the secondary PCR. This number of cycles may have had the effect of increasing background products, which may have led to an overabundance of short fragments and false positives (Diatchenko *et al.,* 1999). Diatchenko *et al.* (1999) recommended 10 to 15 cycles of amplification for the secondary PCR. The primer annealing temperature and elongation temperature used in the PCR programme may also need to be optimised in order to decrease the generation of background products.

4.3.6 Cloning

Cloning of the resulting subtractive hybridisation fragments was necessary in order to generate a sufficient amount of material to sequence. The disadvantage with cloning is that it only allows a sample of the overall DNA fragments, that were obtained by the subtractive hybridisation, to be isolated and identified. It may be possible that many potentially interesting fragments were present but were not isolated by cloning. However, the results showed that certain sequences, such as those corresponding to *flgE*, were isolated more frequently. This would suggest an overabundance of these sequences within the resulting pool of fragments. Due to the short nature of the sequences isolated, this may be explained by the preferential PCR amplification of short DNA fragments over longer ones. Short DNA fragments can also be preferentially cloned due to more efficient ligation into the cloning vector PGEMT-Easy (Diatchenko *et al.,* 1999).

4.4 RAPD-PCR detection of potential virulence determinants

The lack of diversity in the fragments generated by RAPD-PCR made the technique an attractive means of isolating differences between strains with potential importance in virulence. The results presented in Section 3.2 showed different RAPD-PCR profiles were generated for NCTC11168 and F38011. Profiles consisted of two to five bands, depending on the combination of primers used (Figures 3.8 and 3.9). The downfall of this procedure was the lack of reproducibility of the profiles generated (Figure 3.10). In this study, DNA was isolated using the guanidium thiocyanate DNA extraction protocol. It was found that using DNA from two different DNA preparations considerably altered the RAPD profiles that were observed. As other variables of the reaction were kept constant, eg., concentrations of $MgCl_2$, dNTPs, primers and *Taq* polymerase, and the thermocycler and programme, it was reasoned that slight variances in DNA concentration must have been responsible. Welsh and McClelland (1990) reported changes in profiles with changes in DNA concentration. They suggested this may be due to a reduced probability of amplification initiation, reflecting inefficient priming events.

Some researchers have suggested that purified genomic DNA may produce more reproducible profiles than DNA obtained by whole cell lysis techniques (Brikun *et al.*, 1994). However, the results from this study have shown that purified genomic DNA may alter between samples, thereby influencing profiles. Phenol:chloroform extraction of the DNA, to remove any potentially contaminating cell debris, and careful measurement of DNA concentration may have improved reproducibility. Whole cell lysis has been shown to generate reproducible RAPD profiles, where suspensions of bacterial cells were standardised (Armstrong, 1997). The primer annealing temperature may also require optimisation. Raising the annealing temperature in the PCR from 25°C may have made the profiles more robust. With further experimentation and optimisation the reproducibility of the procedure may have improved. However, restricted time did not allow this.

Due to the RAPD profiles generated in this study not being reproducible between DNA samples isolated from the same strain, it is difficult to conclude that differences in profiles

between strains were true differences. The aim of the RAPD experiment was to determine if identical bands from profiles from the two different strains were the same, and whether bands that were different represented true differences between strains. This would have been deduced by eluting the respective bands from the agarose gel, and using Southern hybridisation to determine if bands were the same or different. Similar experiments have been performed by Misawa *et al.* (1998) and Carvalho *et al.* (2001). As the validity of any experiment is subject to experimental reproducibility, it was decided to discontinue this line of work until the problems could be resolved.

4.5 Differential gene expression

Investigating the differential expression of genes between two different strains or two different conditions is a potential means of identifying novel genes. A number of methods have been applied in order to investigate differential gene expression, including mRNA-based subtractive hybridisation and differential-display (Liang & Pardee, 1992; Plum & Clark-Curtiss, 1994). The application of such techniques to elucidate differences in gene expression in *C. jejuni* was initially investigated in this study. The primary step in any mRNA detection protocol is reliable isolation of RNA and reverse transcription of mRNA. Satisfactory controls are also required to ensure validity of any results. These aspects and their potential impact in the subsequent application to the study of differential gene expression are discussed in the following sections.

4.5.1 RNA isolation and experimental controls

On the basis of gel electrophoresis, total RNA isolation using the RNeasy Plant Mini Kit (Qaigen) appeared highly reproducible (results not shown). However, total RNA is not necessarily representative of mRNA in the sample. RNA isolated using this protocol is frequently contaminated with DNA, as evidenced by PCR amplification from a RNA sample subjected to RNaseA digestion (Figure 3.11, lane six). To avoid detection of contaminating

DNA in subsequent reactions, RNA samples were subjected to DNaseI digestion. The results from experiments using conventional PCR detection (Section 3.3) indicated RNA samples treated in this way were mainly free from DNA contamination. However, TaqMan® PCR, a more sensitive method, indicated that DNA contamination was present. This was suggested from the positive amplification of the *ciaB* gene from F38011Δ*ciaB* (0.1% DCA) and F38011Δ*gmhD* (standard conditions) (Figure 3.19). The most likely explanation for these results would be incomplete DNaseI digestion. Because the C_T values for *ciaB* amplification in each of the samples were greater than 30, this possible DNA contamination would not have been detected using conventional PCR methods where only 25-30 cycles of amplification were performed. The results indicate that when more sensitive detection methods are used, the controls used to ensure absence of DNA in the sample may not be effective.

Had this line of work continued to the application of a technique to detect differential gene expression, DNA contamination in the sample may have invalidated results (Hubank & Schatz, 1999). This is primarily due to the manipulation of cDNA being preferable to mRNA, which is generally unstable, in these experimental procedures.

4.5.2 Differential expression controls

In order to test a technique to detect differential gene expression in strains of *C. jejuni* it was first necessary to develop suitable control experiments. A useful method would be able to detect the difference in expression of just one gene. To test this, the experiments investigating induction of *ciaB* (Section 3.3.2) and detection of *gmhD* expression (Section 3.3.3) were performed.

Induction of *ciaB* in the presence of DCA was initially investigated as a means of testing differential expression, due to the expression of this gene in the presence of bile salt and the availability of the isogenic set F38011 and F38011Δ*ciaB*. The gene *ciaB* was not detected by PCR from F38011 cultures grown in the presence of 0.1% DCA. The results suggested

lack of *ciaB* expression under this condition. Pilus production has been shown to be induced in culture with 0.1% deoxycholic acid supplemented to Mueller-Hinton agar (Doig *et al.*, 1996). This indicates 0.1% DCA was sufficient to mimic aspects of the human gut. TaqMan® PCR indicated *ciaB* expression may not have been detected by conventional PCR. However, these results also suggested possible DNA contamination, which could have led to false-positive detection of expression.

The gene *hupB* is constitutively expressed and located immediately downstream from *ciaB* (Konkel *et al.*, 1999a). *hupB* expression was detected in cultures grown in standard conditions and in the presence of DCA. In *C. jejuni*, *hupB* encodes a histone-like DNA-binding protein (Konkel *et al.*, 1999a). The HU protein in *E. coli* is a heterodimer comprised of two homologous components, one of which is encoded by *hupB*, and is involved in several functions including the initiation of DNA replication and homologous recombination (Jaffé *et al.*, 1997; Li & Waters, 1998). HupB is likely to have a similar role in *C. jejuni*. It may be that *hupB* expression was detected by PCR due to a higher transcript abundance than *ciaB*. 23S rDNA expression was also detected. There are three copies of 23S rDNA present in the genome (Parkhill *et al.*, 2000) which would suggest a higher abundance of 23S rDNA transcripts. Detection of *hupB* by PCR was not likely to have been due to DNA contamination, as contamination was not detected in DNase-treated samples by conventional PCR. The results from the *hupB* PCR suggest that *ciaB* was not detected due to low transcript abundance and/or *ciaB* was simply not induced under the conditions provided.

Due to the inability to detect *ciaB* expression, the differential expression of *gmhD* in the isogenic set F38011 and F38011Δ*ghmD*, was investigated. Expression of *gmhD* was not detected from F38011 by PCR, as expected. Nor was *gmhD* detected in RNA and cDNA obtained from an independent source, indicating lack of detection was not solely due to the RNA isolations and RT-PCR performed in this study. Although no probe was available for *gmhD* detection using TaqMan® PCR, the results from the initial experiments indicate that *gmhD* would not have been detected by conventional PCR at the number of

amplification cycles performed. Again, low transcript abundance may be a plausible explanation for lack of detection.

If low transcript abundance is responsible for lack of detection of *ciaB* and *gmhD*, careful consideration of the method applied to deduce differential gene expression between strains of *C. jejuni* would have to made. RAP-PCR has a bias towards sampling more abundant transcripts (Mathieu-Daudé *et al.*, 1999). However, this method may not have been appropriate for use in the initial control experiments as it is best applied when many differentially expressed genes are expected or when there is no *a priori* argument to predict the extent of differential gene regulation. Sampling of rarer transcripts or transcripts from specific genes by RAP-PCR can be overcome by priming at particular motifs or statistically over-represented sequences among a given family of genes (Mathieu-Daudé *et al.*, 1999). Suppression PCR can be exploited in subtractive hybridisation as the suppression effect leads to normalisation of sequence abundance amongst a target cDNA population (Diatchenko *et al.*, 1996). However, this would still be dependent on efficient RT-PCR of rare transcripts.

4.6 Other techniques for the elucidation of virulence genes of *C. jejuni*

Lack of knowledge of mechanisms of pathogenesis in *C. jejuni* has in part been due to a lack of suitable tools for the efficient generation of random mutants that can be tested in relevant biological assays (Bleumink-Pluym *et al.*, 1999; Golden *et al.*, 2000; Colegio *et al.*, 2001). When this study began, no efficient means of generating random insertional mutants in *C. jejuni* had been developed. However, since this study began, two random transposon mutagenesis systems have been reported (Golden *et al.*, 2000; Colegio *et al.*, 2001). These techniques and their possible implications for future research in the study of pathogenicity in *C. jejuni* will be discussed below.

Golden *et al.* (2000) designed and tested an *in vivo mariner*-based transposon mutagenesis system for the production of random insertional mutants of *C. jejuni*. The *mariner*-family transposon *Himar1* shows little insertion site specificity (Akerley *et al.*, 1998). Transposons derived from the *mariner* element *Himar1* have previously been shown to efficiently transpose in *E. coli* and mycobacteria to produce diverse insertion mutations in chromosomal DNA (Rubin *et al.*, 1999). A genomic analysis and mapping by an *in vitro* transposition technique, based on *Himar1* transposition, has been developed for use in naturally competent bacteria (Akerley *et al.*, 1998).

In the study by Golden *et al.* (2000), an electrocompetent strain *C. jejuni* 480, was used. A mini-*mariner* transposon containing an *E. coli/C. jejuni*-compatible chloramphenicol resistance cassette, was delivered via a suicide vector (pOTHM). The vector contained a *Himar1* transposase under the control of a *C. jejuni* promoter, an *E. coli* ampicillin resistance gene and a pUC19-derived origin of replication. The randomness of insertions in the *C. jejuni* chromosome was ascertained by probing *Hin*dIII-digested chromosomal DNA from the transformants with DNA containing the 5′ and 3′ portions of the mini-transposon. Further confirmation was obtained by sequencing. The transposon mutagenesis method used by Golden *et al.* (2000) was shown to generate highly random insertional mutations. Further analysis of the implication of these insertions on the pathogenic phenotype of *C. jejuni* was not performed in the study. Colegio *et al.* (2001) suggested that limitation of the *mariner*-based system was that restriction of the suicide vector was unavoidable and this severely affected its efficiency.

To overcome the limitations of other mutagenesis systems developed for *C. jejuni*, Colegio *et al.* (2001) designed an *in vitro* mutagenesis strategy based on the *S. aureus* transposable element Tn*552*. A derivative of Tn*552* was constructed by replacing a chloramphenicol resistance gene with the kanamycin resistance gene *aphA*-3 from *C. coli*. This derivative was termed Tn*552kan-Campy*. Tn*552kan-Campy* was shown to undergo transposition *in vitro* with a similar efficiency to the parent element. Transformants were identified by growth on kanamycin-containing plates. Electroporation of *C. jejuni* 81-176 produced

between 2.85 x 10^3 and 7.68 x 10^3 kanamycin resistant colonies when Tn*552kan-Campy* was isolated from *C. jejuni*, as opposed to *E. coli* where much lower transformation efficiencies were observed. The randomness of the insertions in *C. jejuni* chromosome were assessed by probing *Bsp*HI-digested chromosomal DNA with labelled Tn*552kan-Campy* DNA. A *Bsp*HI site was present within the transposon, therefore digestion with this enzyme was expected to produce two unique bands of different sizes if the mutants resulted from a single insertion event. Randomness was also confirmed by sequencing. The results of Colegio *et al.* (2001) indicated insertions occurred at different sites of the *C. jejuni* chromosome and Tn*552kan-Campy* did not exhibit any preference towards certain regions. To demonstrate the utility of the transposition system, Colegio *et al.* (2001) screened insertion mutants for motility defects. Nine were found to be non-motile, two of which revealed insertions in genes not previously associated with motility. Colegio *et al.* (2001) demonstrated that Tn*552kan-Campy* is an efficient transposition system for the generation of random mutants. The utility of this system, in combination with the sequenced genome of NCTC11168, has shown its potential in the identification of genes that may be involved in virulence.

The methods outlined above may provide a more efficient means of investigating the pathogenic properties of *C. jejuni* than the subtractive hybridisation technique. It was suggested that the Tn*552*-based transposition system could be adapted for use in signature-tagged mutagenesis (STM) (Colegio *et al.*, 2001). STM is a transposon-based mutagenesis scheme, in which each transposon is marked with a different DNA sequence tag. This technique allows the comparison of mutants that survive passage through an animal with those that survive through culture medium (Cotter & Miller, 1998). STM has been used to search for new virulence genes in *S. typhimurium* and led to the discovery of SPI2, encoding a novel type III secretion system (Hensel *et al.*, 1997). Once the genes that are required by *C. jejuni* for virulence are identified, the ubiquity of the genes within the species, and therefore the pathogenic potential of certain strains, could be assessed.

With these new technologies available, the development of subtractive hybridisation as a means of studying pathogenic potential in *Campylobacter* may not be necessary. This study has shown that subtractive hybridisation would need to be optimised for use in *C. jejuni* in order to demonstrate its utility.

4.7 Conclusions

In this thesis the application of a PCR-based subtractive hybridisation method in *C. jejuni* has been described. This technique, as applied here, does not appear to be an efficient means of isolating differences between strains that may be important in the pathogenicity of this organism. However the results presented suggest subtractive hybridisation was highly reproducible and sequence analysis of a limited number of fragments indicated its potential to isolate differences between strains of *C. jejuni* with relevance to virulence. Further optimisation of the technique is required. However, it may be that substantial heterogeneity between strains of *C. jejuni* and the nature of these differences, ie., variable coding regions and genomic rearrangements, would prevent efficient application of a DNA-based subtractive hybridisation in *C. jejuni*. The method appears most appropriate when a substantial amount of DNA is present in one strain that is absent in another, eg., pathogenicity islands. Thus far, PAI have not been identified in *C. jejuni*, but if they exist, as they do in other enteric pathogens, this study would not rule out subtractive hybridisation as a means of PAI isolation. Applying subtractive hybridisation with this specific task in mind would require thorough screening of strains and would be a long and arduous process.

Techniques investigating differences in gene expression may be more appropriately applied in *C. jejuni* than DNA-based techniques. Because it is differently expressed genes that are isolated and not differences in genomic DNA, these techniques would limit the isolation of background differences such as silent mutations. The application of these techniques was initially investigated in this study but, due to difficulties experienced with RT-PCR detection of specific gene expression, the potential of such techniques could not be fully

realised. Further study in this area may prove more successful than DNA-based techniques in the investigation of virulence genes of *C. jejuni*. A suitable model for induction of genes required *in vivo* must also be sought, as induction of *ciaB* with 0.1% DCA was unsuccessful in this study.

The recent development of efficient transposon mutagenesis techniques may lead the way in the study of virulence determinants in *C. jejuni*. Once more knowledge is gained of the disease-causing requirements of *C. jejuni* it may be possible to gain a better understanding of the pathogenic potential of this important pathogen.

4.8 Future research

Due to the time constraints involved in Master's research, much of the work presented in this thesis remains inconclusive. Further sequence analysis is required to determine the nature of the *mutY* and *sdaA* fragments isolated by the DNA-based subtractive hybridisation. This would enable the elucidation of differences in these genes between the strains NCTC11168 and F38011 and provide conclusive evidence that isolation of these fragments was not due to the generation of false-positive sequences. Primers would be designed, based on the NCTC11168 genome sequence. PCR amplification and subsequent sequencing of these genes from the respective strains would allow any strain differences at these loci to be observed.

As mentioned earlier, the subtractive hybridisation method requires optimisation. This may lead to the isolation of longer sequences with greater differences in nucleotide sequence. The choice of strains used should also be considered. Had any significant differences between strains been isolated, the significance with regards to pathogenicity might have been able to be determined. This would be achieved by introducing an insertional disruption in the appropriate gene, followed by a biological assay to determine a change in phenotype relevant to virulence. Cell binding and internalisation assays using the INT407 or Caco-2

tissue-culture lines would be recommended, as adherence and internalisation in host cells are important virulence determinants. A complementation experiment would also have to be performed to ensure the original phenotype could be restored. In order to gain an overall view of the relevance of the strain difference(s) to the pathogenic potential of different strains of *C. jejuni*, the dissemination of the isolated difference through the greater population could be assessed by PCR and hybridisation experiments. Once the difference was identified in other strains, biological assays would again be used to confirm its role in pathogenicity.

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Appendix I

Media

Unless otherwise stated, all media were sterilised by autoclaving for 20 min, 121°C at 120 kPa. Solutions that could not withstand autoclaving were filter-sterilised by passage through a 0.22 μm filter prior to addition to sterile media.

Brain heart infusion broth (BHI)

	Per litre
Beef heart infusion	25 g
Calf brain infusion	20 g
Protease peptone	10 g
NaCl	5 g
$Na_2HPO:12H_2O$	2.5 g
Glucose	2 g

Dissolved in dH_2O and pH adjusted to 7.4 at 25°C before autoclaving.

Campylobacter blood-free selective agar base (Modified CCDA-Preston)

(Oxoid CM 739)

	Per litre
Beef extract	10 g
Peptone	10 g
NaCl	5 g
Charcoal	4 g
Casein hydrolysate	3 g
Sodium deoxycholate	1 g
Ferrous sulfate	0.25 g
Sodium pyruvate	0.25 g
Bacteriological agar	12 g

Dissolved in dH_2O and pH adjusted to 7.4 at 25°C before autoclaving. Cefoperozone was added to a final concentration of 32 μg/ml before media was poured into sterile Petri dishes.

Luria Bertani (LB) medium

	Per litre
1.0% w/v Bacto tryptone	10 g
0.5% w/v yeast extract	5 g
0.5% w/v NaCl	5 g

Dissolved in dH_2O and pH adjusted to 7.4 with 2M NaOH before autoclaving.

LB agar (LBA)

	Per litre
LB media with addition of 1.5% w/v agar	15g

Mueller-Hinton blood agar (MHA)

	Per litre
Beef infusion	30 g
Acid hydrolysate of casein	17.5 g
Starch	1.5 g
Bacto-agar	17 g
Sheep blood, defibrinated	50 ml

Media was dissolved in dH_2O and pH adjusted to 7.4 at 25°C before autoclaving. The basal media was allowed to cool to 50°C before the addition of sterile defibrinated sheep's blood. Media was poured into sterile Petri dishes and allowed to set before storage at 4°C.

MHA with deoxycholic acid (DCA)

MHA with addition of 0.1% w/v DCA

SOC medium

	Per 100 ml
Bacto®-typtone	2 g
Bacto®-yeast extract	0.5 g
1M NaCl	1 ml
1M KCl	0.25 ml
2M Mg^{2+} stock	1 ml
2M glucose	1 ml

Bacto®-typtone, Bacto®-yeast extract, NaCl and KCl were dissolved in 97 ml dH_2O, autoclaved and allowed to cool to ambient temperature. The 2M Mg^{2+} stock and 2M glucose were added to the medium (to a final concentration of 20mM) and the total volume was adjusted to 100 ml with sterile dH_2O. The complete medium was filter-sterilised by passage through a 0.2 μm filter. The final pH was 7.0.

2M Mg^{2+} stock

	Per 100 ml
$MgCl_2.6H_2O$	20.33 g
$MgSO_4.7H_2O$	24.65 g

Dissolved in dH_2O and filter-sterilised.

Appendix II

Buffers and Solutions

II.i Common buffers and solutions

Buffers and solutions requiring sterilisation were autoclaved for 20 min at 121°C at 120 kPa, or filter sterilised by passage through a 0.22 μm filter. Unless otherwise stated, all buffers and solutions were stored at ambient temperature.

TE

	Per litre
10 mM Tris-HCl	1.2 g
1 mM EDTA	0.38 g

Dissolved in dH_2O and pH adjusted to 8.0.

0.5M EDTA

	Per litre
$Na_2EDTA:H_2O$	186.1 g

Dissolved in dH_2O and pH adjust to 8.0 with 10M NaOH.

1M Tris

	Per litre
Tris base	121.1g

Dissolved in 800 ml dH_2O and pH adjusted to the desired pH with concentrated HCl. Made up to a final volume of 1 L with dH_2O.

Ethidium bromide (10 mg/ml)

0.2 g ethidium bromide dissolved in 20 ml dH_2O. Stored in the dark at 4°C.

50× TAE

	Per litre
2.5 M Tris base	242 g
0.11% v/v glacial acetic acid	57.1 ml
50 mM Na_2EDTA (pH 8.0)	46.5 g

Dissolved in dH_2O to a final volume of 1 L and pH adjusted to 8.0.

1× TAE

20 ml of 50 x TAE dissolved in dH_2O to a final volume of 1 L.

6× DNA loading dye for agarose gel electrophoresis

50% (v/v) Glycerol

0.25% (w/v) Bromophenol blue

0.25% (w/v) Xylene cyanol

Made up to 10 ml in dH_2O.

10× TBE

	Per litre
0.5M Tris base	108 g
0.5M Boric acid	55 g
10 mM Na_2EDTA	9.3 g

Dissolved in dH_2O to a final volume of 1 L and pH adjusted to 8.0.

II.ii Chromosomal and plasmid DNA purification solutions

7.5M Ammonium Acetate

	Per litre
NH_4Ac	577.5g

Dissolved in dH_2O to a final volume of 1 L.

Chloroform:Isoamyl Alcohol

	Per 100 ml
Chloroform	96 ml
Isoamyl alcohol	4 ml

Mixed thoroughly and stored at 4°C.

3M Sodium Acetate

	Per litre
$NaAc:3dH_2O$	408 g

Dissolved in dH_2O to a final volume of 1 L and pH adjusted to 5.2 with glacial acetic acid.

GES lysis solution

	Per 100 ml
Guanidium thiocyanate	60 g
0.5 M EDTA, pH 8.0	20 ml
ddH_2O	20 ml
10% v/v N-lauroyl sarcosine	5 ml

Guanidium thiocyanate and ddH_2O heated to 65°C and mixed until dissolved. The mixture was cooled before the addition of 10% N-lauroyl sarcosine. Made up to 100 ml with ddH_2O and passed through a 0.45 μm filter.

5M Sodium Chloride

	Per litre
NaCl	295 g

Dissolved in 800 ml dH_2O by heating. Made up to 1 L final volume.

STET

	Per litre
1M NaCl	100 ml
1M Tris.Cl, pH 8.0	100 ml
10mM EDTA, pH 8.0	100 ml
5% Trition X-100	50 ml

Dissolved in dH_2O to a final volume of 1 L.

II.iii Southern hybridisation solutions

Depurination solution

	Per litre
0.5M NaOH	20 g
1.5M NaCl	87.7 g

Dissolved in ddH_2O.

Neutralisation solution

	Per litre
0.5M Tris-HCl, pH 7.0	76.9 g
3M NaCl	175.5 g

Dissolved in ddH_2O and pH adjusted to 7.0.

20× SSC (Transfer solution)

	Per litre
3M NaCl	175 g
0.3M Trisodium citrate	88 g

Dissolved in ddH_2O and pH adjusted to 7.0.

Maleic acid buffer

0.1M Maleic acid

0.1M NaCl

pH adjusted to 7.5

10× Blocking solution

10% v/v Blocking reagent

90% v/v Maleic acid buffer

Prepared freshly for each Southern hybridisation.

Post-hybridisation low stringency wash I

2× SSC

0.1% SDS

Post-hybridisation low stringency wash II

0.5× SSC

0.1% SDS

Detection buffer

0.1M Tris-HCl, pH 9.5

0.1M NaCl

II.iv Subtractive hybridisation solutions

5× Hybridisation buffer

2.5M NaCl

250mM HEPES, pH 8.3

1mM EDTA

1× Hybridisation buffer

20% v/v 5× Hybridisation buffer

Dissolved in ddH_2O.

Appendix III

Blastx Results

For all blastx alignments, the query refers to the amino acid sequence corresponding to the sequenced subtractive hybridisation fragments. The subject refers to the amino acid sequence from the NCTC1168 sequenced genome available in Genbank. All blast searches were performed via the NCBI website (www.ncbi.nlm.nih.gov/BLAST/).

III.i Blastx alignment for isolated *flgE* fragments

>gi|11346589|pir||E81271 flagellar hook chain protein Cj1729c [imported] - Campylobacter jejuni (strain NCTC 11168)

gi|6969144|emb|CAB73715.1| (AL139079) flagellar hook subunit protein [Campylobacter jejuni]

Length = 865

Score = 36.2 bits (82), Expect = 0.075

Identities = 16/16 (100%), Positives = 16/16 (100%), Frame = +1

```
Query: 1   DQTIDSSRTPQNIFID 48

           DQTIDSSRTPQNIFID

Sbjct: 145 DQTIDSSRTPQNIFID 160
```

III.ii Blastx alignments for isolated *mutY* fragments

>gi|11346550|pir||B81258 A/G-specific adenine glycosylase (EC 3.2.2.-) Cj1620c [imported] - Campylobacter jejuni (strain NCTC 11168)

gi|6969037|emb|CAB73608.1| (AL139079) A/G-specific adenine glycosylase [Campylobacter jejuni]

Length = 339

Score = 49.3 bits (116), Expect = 9e-06

Identities = 22/22 (100%), Positives = 22/22 (100%), Frame = -1

```
Query: 67  DLKKLSGIGAYTAGAIACFGYD 2

           DLKKLSGIGAYTAGAIACFGYD

Sbjct: 126 DLKKLSGIGAYTAGAIACFGYD 147
```

>gi|7520859|pir||F71335 probable A/G-specific adenine glycosylase - syphilis spirochete

gi|3322622|gb|AAC65331.1| (AE001214) A/G-specific adenine glycosylase, putative [Treponema pallidum]

Length = 277

Score = 36.2 bits (82), Expect = 0.075

Identities = 14/22 (63%), Positives = 18/22 (81%), Frame = -1

```
Query: 67  DLKKLSGIGAYTAGAIACFGYD 2

           +LKKL G+G YTA A+ACF Y+

Sbjct: 117 ELKKLPGVGDYTAAAVACFAYN 138
```

>gi|4467625|emb|CAB37764.1| (AJ239653) MutY protein [Helicobacter pylori]

Length = 140

Score = 34.7 bits (78), Expect = 0.22

Identities = 15/21 (71%), Positives = 17/21 (80%), Frame = -1

```
Query: 67  DLKKLSGIGAYTAGAIACFGY 5

           +L KL GIGAYTA AI CFG+

Sbjct: 60  NLLKLPGIGAYTANAILCFGF 80
```

III.iii Blastx alignment for isolated *sdaA* fragment

>gi|11269014|pir||F81258 L-serine dehydratase (EC 4.2.1.13) Cj1624c [imported] - Campylobacter jejuni (strain NCTC 11168)

gi|6969041|emb|CAB73612.1| (AL139079) L-serine dehydratase [Campylobacter jejuni]

Length = 454

Score = 45.4 bits (106), Expect = 1e-04

Identities = 20/20 (100%), Positives = 20/20 (100%), Frame = +3

```
Query: 3   ANAFKACNAAEMAMEHHLGL 62

           ANAFKACNAAEMAMEHHLGL

Sbjct: 359 ANAFKACNAAEMAMEHHLGL 378
```